

Inventor(s): John C. Reed
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REMARKS

Claims 11-14, 16, 20-27, 32-34, 36, 37, 44 and 50-66 are pending in the present application. Claims 16, 20, 25, 27, 34 and 44 have been amended herein. Following entry of the amendments, claims 11-14, 16, 20-27, 32-34, 36, 37, 44 and 50-66 will remain under examination.

Regarding the claim amendments

Claims 16, 20, 25, 27 and 34 have been amended to indicate that samples useful in the methods include a sample of tumor cells from a body fluid. These amendments are supported in the specification, for example, at page 8, lines 15-18, which indicates that cancerous cells of a patient "may be drawn from a body fluid such as lymphatic fluid, blood, serum, or a distally infected organ or exudate thereof" in the case of metastatic cancer.

Claims 16, 25, 34 and 44 have been amended to recite levels of expression of BAG-1 relative to a reference level of BAG expression. These amendments are supported in the specification, for example, at page 16, lines 13, to page 17, line 2, which indicates that a "high level" of BAG gene expression is related to a level of BAG gene expression above a determined basal level.

As set forth above, the claim amendments do not add new matter. Therefore, Applicant respectfully requests that the Examiner enter the amendments.

Rejections under 35 U.S.C. § 112, first paragraph

The objection to the specification and corresponding rejection of claims 6-8, 11-14, 16, 18-27, 29-37 and 44 under 35 U.S.C. 112, first paragraph, as allegedly lacking enablement is respectfully traversed. Applicant submits that the full scope of these claims is enabled by the specification.

Regarding body fluids

The Office Action alleges that the specification lacks enablement for methods in which BAG-1 protein expression levels are measured in a body fluid. Applicant respectfully submits that one skilled in the art would have been able to practice the claimed methods using a sample that is body fluid in much the same way as when using a tumor sample. In this regard, the specification provides teachings that a body fluid is a suitable sample for use in the claimed methods (see, for example, page 8, lines 9-24); a rationale for why a body fluid is a suitable sample (see, for example, page 8, lines 18-23); provides procedures for performing steps of the claimed methods (see, for example, page 13, lines 21-29); and working examples of measuring a level of BAG-1 protein expression and correlating levels of BAG-1 protein expression with clinical outcome (see, for example, page 35, line 5, to page 36, line 6; and page 33, lines 25, to page 34, line 16). In view of these teachings in the specification, Applicant submits that one skilled in the art would have been able to use the claimed methods without undue experimentation.

Nevertheless, as amended, claims 16, 20, 25, 27 and 34 recite a sample containing tumor cells from a bodily fluid. Applicant submits that the specification provides enablement for methods in which BAG-1 protein expression levels are measured in tumor cells from a body fluid. In particular, the specification teaches that cancerous cells from a patient's tumor can be obtained from a body fluid (page 8, lines 15-18). Given that tumor cells contained in the body fluid originate from a tumor, one skilled in the art would understand that BAG-1 expression data obtained using tumor cells obtained from either a sample of a tumor or a sample of tumor cells from a body fluid would yield consistent prognostic results. For this reason, Applicant submits that undue experimentation would not have been required to practice the claimed methods using both a sample of a tumor and cells from the tumor.

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Regarding reference levels

The Office Action alleges that methods involving determination of a reference level of BAG-1 protein lack enablement in the specification because the specification lacks description of an actual measurement of BAG-1 protein useful for discerning a "high" level of BAG-1 protein. It is however acknowledged in the Office Action that the specification indeed exemplifies determination of an H-score useful for discerning a "high" level of BAG-1, but whether the determined H-score can be used in any study of breast tumor samples is questioned.

Applicant respectfully submits that the specification and publications available at the time of filing the present application provide a reasonable amount of guidance to allow one skilled in the art to determine a reference level for use in the claimed methods. Using this guidance, one skilled in the art would have practiced only routine methods to determine an H-score or other reference level using a selected anti-BAG antibody and detection system. The specification teaches, for example, on page 18, line 25, to page 19, line 24, that a reference level can be determined by comparing patient populations:

The reference level may also be determined by comparison of BAG expression levels in populations of patients having the same cancer. This may be accomplished by histogram analysis, in which the entire cohort of patients tested are graphically presented, wherein a first axis represents the level of BAG expression, and a second axis represents the number of patients in the cohort whose tumor cells express BAG at a given level. Two or more separate groups of patients may be determined by identification of subsets populations of the cohort which have the same or similar expression levels of BAG. Determination of the reference level may then be made based on an expression level which best distinguishes these separate groups.

As further evidence that determination of a reference level for a prognostic protein marker was routine at the time of filing the present application, Applicant submits herewith Borre et al. Prostate Cancer and Prostatic Diseases 5:268-275 (1998) (Exhibit A) and Kim et al. Cancer Lett. 132(1-2):91-97 (1998) (Exhibit B). Both of these references describes established

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methods for determining a reference level of a prognostic protein. These and a multitude of other publications relating to the use of protein expression for prognostic applications were available to those skilled in the art prior to the filing date of the present application.

Specifically regarding use in the claimed methods of the H-score set forth in the specification (see page 33, line 33, to page 34, line 13), Applicant points out that only routine experimentation would have been required for one skilled in the art to determine an H-score using assay reagents different from those described in the specification, such as a different anti-BAG antibody or detection system. That undue experimentation would not have been needed to determine a reference level is supported by (a) guidance provided in the specification for determining a reference level (page 17, line 31, to page 22, line 4); (b) the presence of working examples of determining an H-score (see page 33, line 33, to page 34, line 13); (c) the high level of skill in the art for determining H-scores (see Exhibit C: Budwit-Novotny et al., Cancer Research, 46: 5419-5425 (1986)) and (d) the predictability of determining that high levels of BAG-1 expression relative to a reference level of BAG-1 expression correlates with survival in view of the teachings in the specification (page 35, line 24, to page 26, line 6).

Given the guidance provided in the specification and well known procedures for determining a reference level of protein expression for use in a prognostic method, only routine methods would have been required for one skilled in the art to practice the claimed invention. For this reason, Applicant requests removal of this rejection under 35 U.S.C. 112, first paragraph.

Regarding individuals having breast cancer

The Office Action alleges that the specification lacks enablement for methods for prognosis of disease-free survival or overall survival of an individual having breast cancer. Specifically, the Office Action alleges that the specification lacks description of methods for prognosis of disease-free survival of an individual already having breast cancer. Paradoxically, the Office Action acknowledges that the specification teaches methods for the prognosis of

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disease-free survival of an individual who will be treated for breast cancer. Applicant respectfully points out that an individual who will be treated for breast cancer is also an individual who already has breast cancer. Applicant points out that the specification exemplifies use of the claimed prognostic method using samples derived from 116 women already having early-stage breast cancer (page 32, lines 11-18). Whether or not the patients received cancer therapy was not relevant to the observation that a high level of BAG-1 expression correlates with survival. In particular, as is described on page 35, lines 5-14, patients having low levels of BAG-1 expression experienced less survival in spite of receiving cancer therapy. Given that the specification exemplifies a correlation between high levels of BAG-1 expression and survival of breast cancer patients having stage I or II cancer, Applicant submits that one skilled in the art would have practiced the claimed methods without undue experimentation. For this reason, Applicant requests removal of this rejection under 35 U.S.C. 112, first paragraph.

Rejection under 35 U.S.C. § 112, second paragraph

The rejection of claims 11-14, 16, 20-27, 32-34, 36, 37, 44, and 50-66 under 35 U.S.C. § 112, second paragraph, as allegedly indefinite is respectfully traversed.

The Office Action alleges that the rejected claims are indefinite for reciting a “high” or “low” level of BAG-1 without recitation of a reference level above which or below which a level can be considered “high” or “low.”

Applicant respectfully submits that the scope of the rejected claims would be reasonable ascertainable by those skilled in the art because the specification teaches the meaning of “high” and “low” levels of BAG-1 expression and provides ample guidance to those skilled in the art for selecting appropriate reference levels for use in the claimed methods. Specifically, the specification teaches that a “high” level of BAG-1 expression means a level of BAG gene expression above a determined basal, or reference, level (page 16, line 31 to page 17, line 2), and that a “low” level of BAG-1 means a level of BAG gene expression below the determined reference level (page 16, lines 27-30). Further, one skilled in the art recognizes that a specific

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value for a reference level can vary depending on the assay system used. For this reason, delineation of a specific value for a recitation of a specific value for a reference level above which or below which a level can be considered "high" or "low" would not have been required for practice of the claimed invention. In view of the above, Applicant requests removal of this rejection under 35 U.S.C. § 112, second paragraph.


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CONCLUSION

In light of the amendments and remarks herein, Applicant submits that the claims are now in condition for allowance and respectfully request a notice to this effect. Should the Examiner have any questions, he is invited to call the undersigned attorney.

Respectfully submitted,

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Immunohistochemical BCL-2 and Ki-67 expression predict survival in prostate cancer patients followed expectantly

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The prognostic value of BCL-2 expression, solely and combined with Ki-67 expression, was determined in prostate cancer patients followed expectantly. Furthermore, associations with well established prognostic markers were tested. Formalin fixed, paraffin-embedded tumour tissue obtained at diagnosis was immunohistochemically investigated in 221 patients with a 15 y median follow-up time. BCL-2 protein was expressed in 114 (52%) tumours and was significantly associated with tumour stage ($P=0.01$). The prognostic value of BCL-2 expression was significant, using both disease-specific ($P=0.0015$) and overall survival ($P=0.005$) as endpoint. Patients with a combined BCL-2 negative/Ki-67 'low' tumour had the most favourable prognosis. This combined BCL-2/Ki-67 variable was of independently prognostic value in both the entire population ($P=0.0001$) and in the clinically localized subpopulation ($P=0.035$).

Keywords: BCL-2; prognostic marker; prostate cancer; apoptosis; proliferation; Ki-67

Introduction

Prostate cancer is the second most commonly diagnosed non-skin cancer disease in Danish males and the second leading cause of cancer death in the same population.^{1,2} Although prostate cancer has been regarded as a slow-growing, not very aggressive tumour, it is well known that some tumours have an aggressive feature.^{3,4} Therefore, there exists an urgent need to identify prognostic markers that can predict with reasonable accuracy the rate of progression of a prostate cancer as well as the response to particular interventions.

The net tumour growth rate is determined by the balance between the concurrently occurring cell proliferation and cell death in tumour cell populations.⁵ The tumour cell proliferation rate can be determined by the mouse monoclonal antibody MIB-1, which is directed against recombinant parts of the Ki-67 antigen present in the G1-, S-, G2- and M-phases of the cell cycle.⁶ The prognostic value of tumour cell proliferation rate (Ki-67) has been demonstrated in several previous studies.⁷⁻¹² Apoptosis, the genetically programmed mode of cellular

suicide, which culminates in nuclear fragmentation and cell death, maintain tissue homeostasis.¹³ Abnormalities in the level of apoptosis, allowing the survival of mutated cells, can lead to clonal expansion of preneoplastic cells.¹⁴ BCL-2 is an inner mitochondrial membrane protein, which main effect is to prolong cell survival by avoidance of apoptosis.^{15,16} BCL-2 is normally expressed in a variety of cell types, including neurons, memory lymphocytes cells, as well as the regenerating stem cells that line the basement membrane in glandular epithelium in for example the prostate.¹⁷ Furthermore, BCL-2 expression has been observed in a variety of human cancers.¹⁸⁻²⁴

The aim of this study was to determine the prognostic value of immunohistochemical BCL-2 expression solely and in combination with Ki-67 in prostate cancer patients subjected to watchful waiting. Moreover, associations between BCL-2 expression and well established prognostic markers were tested.

Materials and methods

Patients

In a five year period (01.01.79-31.12.83), 719 residents in Aarhus County, Denmark, made up the total number of men diagnosed with prostate cancer. The patients were

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identified by the Danish Cancer Registry and retrospectively followed from the time of diagnosis until death. Information on the exact date and cause of death originated from death certificates and hospital charts. The retrospectively restaging of the T- and M-classifications (UICC 1992) have previously been described in details.⁴ The original histopathological grade (WHO 1980) was used. The patients were followed expectantly and palliated at symptoms only. Consequently, 108 (49%) patients with advanced and/or metastatic disease were treated by either bilateral orchiectomy or administration of oestrogen. Detailed accounts of this cancer population has been published previously.^{4,25}

Exclusion of patients diagnosed at autopsy, patients without prostate surgery at diagnosis, and patients with incomplete data registration made archival tumour tissue theoretically available in 280 of the patients. Unfortunately, tumour tissue in an additional 59 cases was lost.⁴

Specimens

The sections for the immunostaining procedures were retrieved from the formalin fixed, paraffin-embedded tissue used for the original histopathological grading after transurethral resection or prostatectomy in previously untreated patients. The tissue was stored as paraffin blocks since operation, and to avoid loss of antigen immunoreactivity,²⁶ the sections were not stored unstained on glass slides. One representative section per patient was chosen.

Immunohistochemistry

After deparaffinization in four 4-minute changes of petroleum, the sections were rehydrated through graded ethanol series to distilled water. The sections were then subjected to antigen retrieval by heating the slides in three 5-minute periods in a microwave oven at 650 W in citrate buffer (10 mM; pH 6) with replacement of evaporated buffer between periods of heating. Endogenous peroxidase activity in the tissue sections was blocked with 35% hydrogen peroxide in distilled water (1:20) for 20 min.

The primary monoclonal antibody reacting against the BCL-2 protein (clone 124, code no. M0887, IgG1, kappa, Dako, Denmark) was applied to the sections overnight at 4°C at a 1:100 dilution in Tris PBS supplemented with 1% BSA.

Immunohistochemistry, against the Ki-67 antigen, was performed using a monoclonal MIB-1 antibody (DIA-NOVA, no. dia 505, clone MIB-1, mouse IgG1, 1:100) for 30 min at room temperature.^{7,27}

Immunostaining was carried out by use of the Streptavidin-biotin-peroxidase enzyme complex (Dako StreptABCComplex/HRP Duet, Mouse/Rabbit, Code no. K0492), and the chromogen used was Carbazol (Sigma A-5754). Finally the sections were lightly counterstained with 50% haematoxylin.

The positive control sections were cut from normal human tonsil. Furthermore, normal basal cells and lymphocytes in the sections acted as a BCL-2 positive internal control. The negative control sections were performed by substituting the primary antibody with non-specific mouse IgG (Dako, Denmark, code no. X 0931).

Quantitation

The distribution of both BCL-2 and Ki-67 immunoreactivity were heterogeneous and there existed a broad variation in staining intensity. Therefore, areas of highest immunoreactivity ('hot spots') were identified by scanning the tumour sections at low magnification ($\times 40$ and $\times 100$). Tumour cell counts were performed by random sampling technique at $400\times$ using a 10×10 grid (0.0625 square mm) in the eye-piece. The blinded procedures were done by a single observer (MB) unaware of the clinical outcome.

BCL-2 staining was considered positive if unequivocal red staining was seen in the tumour cell cytoplasm, and the immunoreactivity was scored semi-quantitatively as the immunoreactive percent of cells a 'hot spot': 0-4 (0=no immunoreactivity, 1+=approximately 1-25%, 2+=approximately 26-50%, 3+=approximately 51-75%, 4+=approximately 76-100%). Correspondingly, the intensity of the BCL-2 immunoreactivity was categorized from 0 to 3+ compared with the intensity of the lymphocytes (0=no positive staining, 1+=weak intensity, 2+=moderate intensity, 3+=strong intensity).

The Ki-67 score represented the number of stained tumour cell nuclei expressed as a percentage of the total number of at least 500 tumour cell nuclei counted inside 'hot spot' areas.⁷ The intraobserver and the interobserver variability of the quantitation method described were tested in a previous study²⁶ concerning tumour cell proliferation in 45 prostate cancer patients, showing that the correlation coefficients were respectively 0.94 ($P < 0.001$) and 0.91 ($P < 0.001$). In the same study the intertumoural variability was demonstrated significantly higher than the intratumoural variability ($P < 0.001$) by two independent observers.²⁷

Statistics

Statistical analysis was performed using the SPSS 6.1 for Windows (SPSS Inc., Chicago, IL) program package. The two-sided chi-square test was used to test for an association between categorical data. Survival functions were calculated according to the method of Kaplan and Meier and the differences between the survival curves were tested by the log rank test. The Cox proportional hazards regression model was used to analyse the prognostic value of the clinical and laboratory characteristics determined at the time of diagnosis. Disease-specific death (all deaths caused directly by prostate cancer excluding deaths from coexisting disease, accidents and unknown causes) and overall death were used as endpoints. Two-sided P -values less than 0.05 were considered statistically significant.

Results

Patients

The median age at diagnosis was 75 y (range 49-95 y). Only 6 (2%) patients were still alive at the end of the registration period, and according to the hospital charts and death certificates 125 (57%) patients died from

Table 1 Clinicopathological characteristics and BCL-2 expression at diagnosis in the present 221 patients

	Population		BCL-2		P-value
	Original ^a n (%)	Current n (%)	Negative n (%)	Positive n (%)	
Total	719 (100%)	221 (100%)	107 (48%)	114 (52%)	
T-class:					
T1a	45 (6%)	21 (10%)	14 (13%)	7 (6%)	0.03
T1b	166 (23%)	83 (38%)	47 (44%)	36 (32%)	
T2	90 (13%)	27 (12%)	11 (10%)	16 (14%)	
T > 2	367 (51%)	90 (40%)	35 (33%)	55 (48%)	
Tx	51 (7%)				
M-class:					
M0	306 (42%)	161 (73%)	82 (77%)	79 (69%)	0.22
M1	240 (33%)	60 (27%)	25 (23%)	35 (31%)	
Mx	173 (24%)				
Clinical stage:					
T1-2,M0	224 (31%)	125 (57%)	70 (65%)	55 (48%)	0.01
T > 2 and/or M1	418 (58%)	96 (43%)	37 (35%)	59 (52%)	
Unknown	77 (11%)				
Grade:					
Well	142 (20%)	59 (27%)	31 (29%)	28 (25%)	0.76
Moderate	184 (26%)	90 (41%)	42 (39%)	48 (42%)	
Poor	171 (24%)	72 (32%)	34 (32%)	38 (33%)	
Unknown	222 (30%)				
Ki-67 score:					
Low (< median score)	—	111 (50%)	60 (56%)	51 (45%)	0.09
High (> median score)	—	110 (50%)	47 (44%)	63 (55%)	

^aOriginal complete prostate cancer population.⁴

prostate cancer, while 90 (41%) patients died from other causes. One-hundred and twenty-five (57%) patients were diagnosed with clinically localized (T1-2, Nx, M0) disease. Table 1 compares the clinicopathological characteristics at diagnosis of the original 719 patients and the present subpopulation.

BCL-2 expression

The tumours expressed BCL-2 protein in 114 (52%) patients (Table 1). In 13 cases the tumour immunoreactivity were categorized as 1+, 10 cancers as 2+, 22 cancers as 3+, while 69 cancers were categorized as 4+. The staining intensity was scored as 1+ in 73 tumours, 2+ and 3+ in 29 and 12 tumours respectively. However, neither the differentiation of positive BCL-2 expression (1-4+) nor staining intensity (1-3+) provided additional information to that already given by a simple separation of the tumours as being BCL-2 expressing positive or negative. Consequently, in the following text BCL-2 refers to negative or positive expression only.

BCL-2 expression was significantly associated with T-classification ($P=0.03$) and tumour stage (clinically localized versus clinically advanced; $P=0.01$), while no such association with either M-classification ($P=0.2$) or histopathological grade ($P=0.8$) was found. The proliferation rate expressed by the Ki-67 score was categorized as 'low' or 'high' by the median Ki-67 value: total population: 10.3% (range 0-46.1%); clinically localized patients: 8.2% (range 0-27.9%).⁷ As shown in Table 1, no significant association between BCL-2 expression and tumour cell proliferation ('low' vs 'high'; $P=0.09$) existed.

Patients diagnosed with BCL-2 expressing tumours had a significantly shorter disease-specific ($P=0.0015$;

Figure 1) as well as overall survival ($P=0.005$) compared to patients with BCL-2 negative tumours. However, focusing solely at the 125 clinically localized cancer patients significance was not achieved ($P=0.15$).

BCL-2 expression and tumour proliferation

The Kaplan-Meier plots in Figure 2 illustrate the significant ($P<0.0001$) prognostic advantage of the 60 (27%) patients harboring a combined BCL-2 negative/slowly proliferating (Ki-67 'low') tumour compared to the patients suffering from BCL-2 expressing and/or highly proliferating tumours. The classic prognostic markers in the four different BCL-2/Ki-67 groups were compared in Table 2. BCL-2 expression, as an isolated variable, was not a prognostic marker in clinically localized cancer, however, the combined BCL-2 negative/Ki-67 'low' tumour patients had a significantly (0.035) longer disease-specific survival than patients suffering from other BCL-2/Ki-67 tumour combinations (Figure 3).

Multivariable analyses

Table 3 presents 4 Cox multivariable regression analyses including (A) classic prognostic markers, BCL-2 and Ki-67 expression, while the four different BCL-2/Ki-67 combinations were additionally included in the B analyses. The combined BCL-2 negative/Ki-67 'low' variable ($P=0.0001$), T-classification ($P<0.0001$) and histopathological grade ($P=0.03$) were all independent prognostic markers in the entire population using disease-specific death as endpoint. However, the combined BCL-2 negative/Ki-67 'low' variable was the only one reaching significance ($P=0.0003$) in the clinically localized subpopulation.

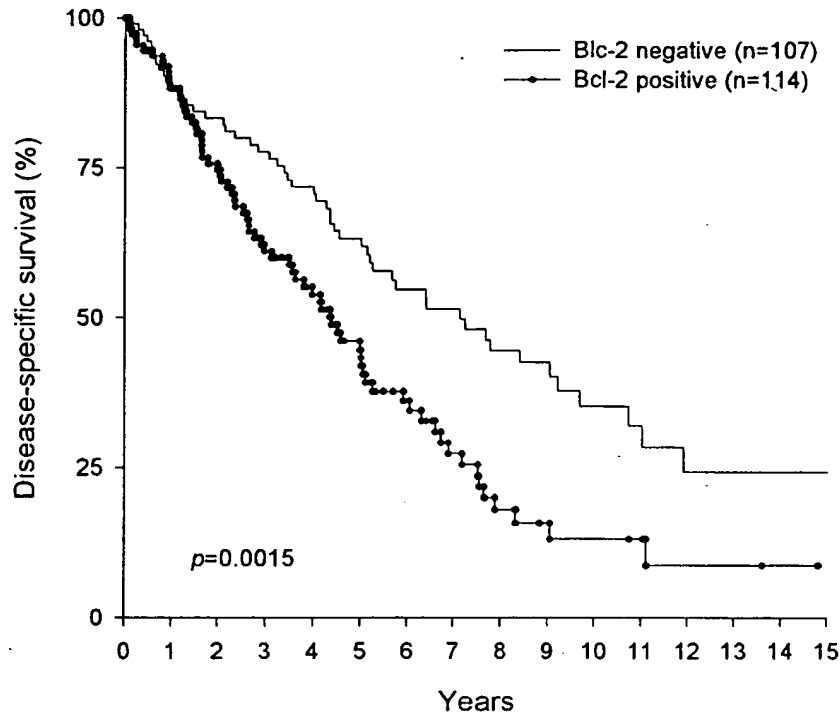


Figure 1 BCL-2 expression at diagnosis correlated with disease-specific survival in 221 prostate cancer patients.

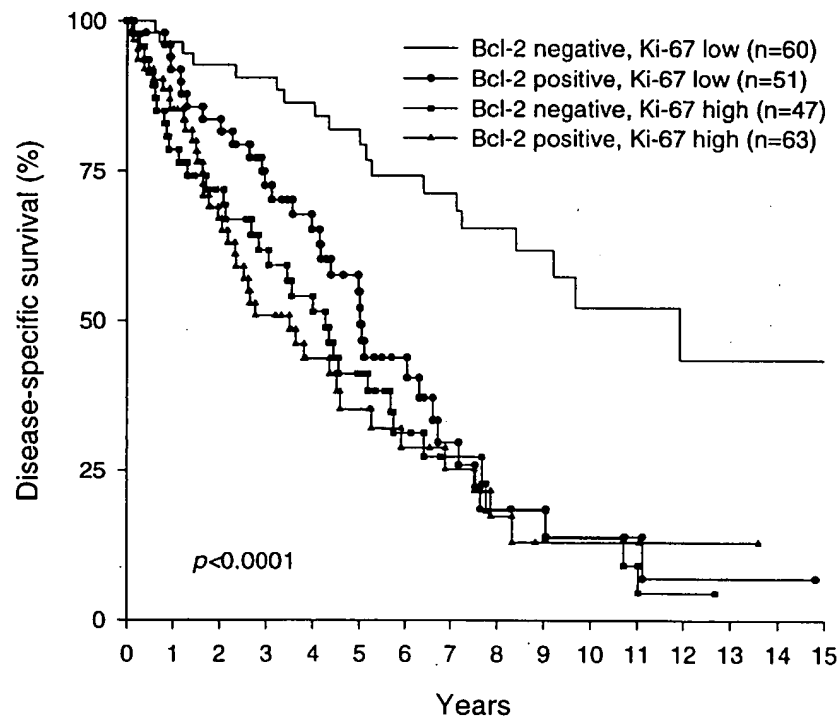


Figure 2 Combined BCL-2 and Ki-67 expression at diagnosis in 221 prostate cancer patients correlated with disease-specific survival. Ki-67: 'low' < median score (10.3%); 'high' > median score.

Table 2 Clinicopathological characteristics compared with four different BCL-2/Ki-67 expression combinations at diagnosis in 221 prostate cancer patients

	BCL2-/Ki-67 low n (%)	BCL2+/Ki-67 low n (%)	BCL2-Ki-67 high n (%)	BCL2 + /Ki-67 high n (%)	P-value
Total	60 (27%)	51 (23%)	47 (21%)	63 (29%)	
T-class:					
T1a	12 (20%)	4 (8%)	2 (4%)	3 (4%)	0.00003
T1b	30 (50%)	25 (49%)	17 (36%)	11 (18%)	
T2	6 (10%)	5 (10%)	5 (11%)	11 (18%)	
T > 2	12 (20%)	17 (33%)	23 (49%)	38 (60%)	
M-class:					
M0	50 (83%)	42 (82%)	32 (68%)	37 (59%)	0.006
M1	10 (17%)	9 (18%)	15 (32%)	26 (41%)	
Clinical stage:					
T1-2,M0	46 (77%)	34 (67%)	24 (51%)	21 (33%)	0.00001
T > 2 and/or M1	14 (23%)	17 (33%)	23 (49%)	42 (67%)	
Grade:					
Well	27 (45%)	17 (33%)	4 (8%)	11 (18%)	0.0005
Moderate	19 (32%)	22 (43%)	23 (49%)	26 (41%)	
Poor	14 (23%)	12 (24%)	20 (43%)	26 (41%)	

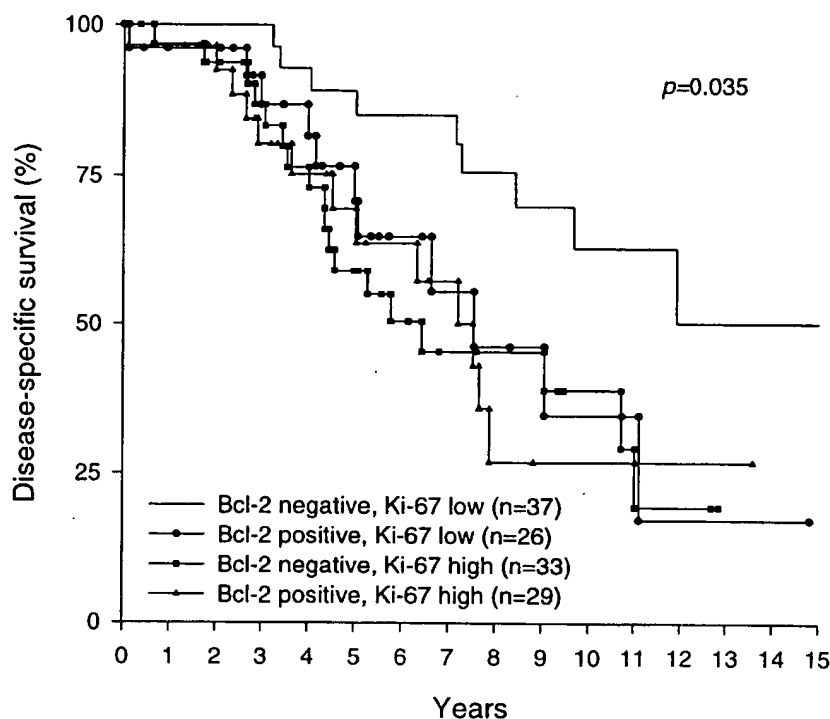


Figure 3 Combined BCL-2 and Ki-67 expression at diagnosis in 125 clinically localized prostate cancer patients correlated with disease-specific survival. Ki-67: 'low' < median score (8.2%); 'high' > median score.

Discussion

The current results were based on a subpopulation originating from a previously described complete prostate cancer population with a nearly complete follow-up subjected to watchful waiting.⁴ This population consists of relatively elderly (median age, 75 y) and often symptomatic prostate cancer patients compared to patients

detected in the PSA era. However, the conservative therapeutic approach towards these patients makes the prognosis information of patients with favourable marker status important. Thereby retrospectively obtained information can be very valuable, although, the clinical information is often incomplete and patients at risk of being understaged. Naturally, retrospective tumour staging is problematic especially with regard to

Table 3 Cox-multivariate regression analyses including traditional markers, BCL-2 expression and Ki-67 score (including combinations) in all 221 prostate cancer patients irrespective clinical stage and in the 125 clinically localized prostate cancer patients using disease-specific death as end-point

	All 221 prostate cancer patients			125 clinically prostate cancer patients		
	P-value	RR	cnf.l. (95%)	P-value	RR	cnf.l. (95%)
A:						
T-class ^a	< 0.0001	1.97	1.60–2.41	0.08		
M-class (M0 vs M1)	0.66			—	—	—
Grade ^b	0.014	1.37	1.06–1.76	0.005	1.62	1.16–2.28
BCL-2 (negative vs positive)	0.10			0.23		
Ki-67 (low vs high)	0.02	1.53	1.06–2.21	0.10		
B:						
T-class (T1a vs T1b vs T2)	< 0.0001	1.96	1.59–2.40	0.12		
M-class (M0 vs M1)	0.37			—	—	—
Grade ^b	0.029	1.32	1.03–1.71	0.071		
BCL-2 (negative vs positive)	0.68			0.22		
Ki-67 (low vs high)	0.91			0.71		
BCL-2/Ki-67 (neg/low vs others)	.0001	0.40	0.24–0.66	0.0003	0.32	0.16–0.62
BCL-2/Ki-67 (pos/low vs others)	0.91			0.71		
BCL-2/Ki-67 (neg/high vs others)	0.68			0.22		
BCL-2/Ki-67 (pos/high vs others)	0.77			0.38		

RR: relative risk. Cnf.l.: confidence limit.

^a (T1a vs T1b vs T2 vs T > 2).^b (well vs moderate vs poor).

a disease with no therapeutic consequences of the accuracy in TM staging.

Although the tumour tissue used for immunohistochemical analyses is relatively old the Ki-67 antigenicity has been shown to be preserved in to previous studies.^{7,27} Moreover normal basal cells and lymphocytes acted as internal positive controls for BCL-2 immunoreactivity. The heterogeneity of prostate cancer is a well established fact,^{28,29} and as expected we observed a heterogeneous distribution of cytoplasmatic BCL-2 immunoreactivity with a variable intensity. For that reason both BCL-2 expression and proliferative rate were quantitated in immunoreactivity 'hot spot' areas of the tumours. In accordance with previous studies examining tumours of various stages^{30,31} our results showed that approximately half of the tumours expressed BCL-2 protein. In general, BCL-2 has been found to be expressed in at most one out of four clinically localized tumours.^{32–34} The relatively high number (48%) of clinically localized BCL-2 expressing tumours in the current study might partly be explained by understaging as well as different limits for positive BCL-2 quantitation among different studies.

As in the current study, an association between BCL-2 expression and clinical stage has previously been demonstrated,^{32–35} while in other studies no such association was found.^{30,31} Moreover, studies of BCL-2 expression in relation to histopathological grade have also been characterized by conflicting results. In accordance with several previous studies,^{30,33,36–39} but in contrast to others,^{31,32,34,35,40} we found no association between BCL-2 expression and histopathological grade ($P=0.8$; Table 1). Thereby, our result supports the suggestion of BCL-2 expression and histopathological grade being two independent prognostic markers in prostate cancer disease.³⁷

We showed, that BCL-2 expression as a univariable marker was significantly correlated with disease-specific

survival in a prostate cancer population of various stages treated without intention to cure (Figure 1). Although, previous studies most frequently have concerned patients subjected to theoretically curable treatment, similar correlations have been demonstrated by others.^{32,33,35} However, Stattin *et al*³¹ only experienced a slight and insignificant association between longer cancer-specific survival and low BCL-2 index in 150 untreated prostate cancer patients. In accordance with some previous studies^{33,35} our multivariable analyses demonstrated an insignificant prognostic value of BCL-2 expression in both the entire population ($P=0.1$) and the clinically localized subpopulation ($P=0.2$).

It is generally accepted that net tumour growth is characterized by a balance between cell proliferation and cell loss rate,⁵ and in a previous study we have demonstrated the significant prognostic relevance of tumour cell proliferation (Ki-67) in the current prostate cancer population.⁷ A transition of late stage high-grade prostatic intraepithelial neoplastic cells into localized prostate cancer cells has been shown not to involve any further increase in proliferation but a decrease in death.⁴¹ In accordance with Bubendorf *et al*³³ the current results showed no significant association between BCL-2 expression and tumour cell proliferation ($P=0.09$) supporting the suggestion, that alterations leading to an increased cell proliferation may be independent from mechanisms affecting the control of programmed cell death. Moreover, like in the Swiss study,³³ the present individuals suffering from a combined BCL-2 negative and slowly proliferative tumours (Ki-67 'low') had a significantly better prognosis than patients with BCL-2 expressing and/or high growth fraction tumours (Figure 2 and 3). This result suggests, that the ability to resist apoptosis does not offer an advantage to rapidly proliferating tumours exclusively, but to tumours characterized by a low proliferation rate as well.

Conclusions

BCL-2 expression was significantly associated with clinical stage, and patients diagnosed with a BCL-2 expressing tumour had a significantly poorer prognosis than BCL-2 negative tumour patients subjected to watchful waiting. Patients with combined BCL-2 negative/Ki-67 'low' tumours had a relatively favorable prognosis, and this BCL-2/Ki-67 expression combination demonstrated an independently prognostic value in both the entire population as well as the clinically localized and theoretically curable subpopulation.

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References

- Engeland A *et al.* Prediction of cancer incidence in the Nordic countries up to the years 2000 and 2010. *APMIS* 1993; 38: 64–66.
- Engeland A *et al.* Prediction of cancer mortality in the Nordic countries up to the years 2000 and 2010. *APMIS* 1995; 49: 84–86.
- Aus G, Hugosson J, Norlén L. Longterm survival and mortality in prostate cancer treated with noncurative intent. *J Urol* 1995; 154: 460–465.
- Borre M, Nerstrøm B, Overgaard J. The natural history of prostate carcinoma based on a Danish population treated with no intent of cure. *Cancer* 1997; 80: 917–928.
- Reed JC. Bcl-2 and the regulation of programmed cell death. *J Cell Biol* 1994; 124: 1–6.
- Cattoretti G *et al.* Monoclonal antibodies against recombinant parts of the Ki-67 antigen (Mib-1 and MIB-3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J Pathol* 1992; 168: 357–363.
- Borre M, Bentzen SM, Nerstrøm B, Overgaard J. Tumor cell proliferation and survival in patients with prostate cancer followed expectantly. *J Urol* 1998; 159: 1609–1614.
- Harper ME *et al.* Pathological and clinical associations of Ki-67 defined growth fractions in human prostatic carcinoma. *Prostate* 1992; 21: 75–84.
- Bettencourt MC *et al.* Ki-67 expression is a prognostic marker of prostate cancer recurrence after radical prostatectomy. *J Urol* 1996; 156: 1064–1068.
- Stattin P, Damber JE, Karlberg L, Bergh A. Cell proliferation assessed by Ki-67 immunoreactivity on formalin fixed tissues is a predictive factor for survival in prostate cancer. *J Urol* 1997; 157: 219–222.
- Raymond WA *et al.* Growth fractions in human prostatic carcinoma determined by Ki-67 immunostaining. *J Pathol* 1988; 156: 161–167.
- Nemeto R *et al.* S-phase fraction of human prostate adenocarcinoma studies with in vivo bromodeoxyuridine labeling. *Cancer* 1990; 66: 509–514.
- Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br J Cancer* 1972; 26: 239–257.
- Raff MC. Social controls on cell survival and cell death. *Nature* 1992; 356: 397–400.
- Hockenbery D *et al.* Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990; 348: 334–336.
- Bonkhoff H. Role of the basal cells in premalignant changes of the human prostate: a stem cell concept for the development of prostate cancer. *Eur Urol* 1996; 30: 201–205.
- Hockenbery DM *et al.* Bcl-2 protein is topographically restricted to tissues characterized by apoptotic cell death. *Proc Natl Acad Sci USA* 1991; 88: 6961–6965.
- McDonnell TJ *et al.* Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. *Cancer Res* 1992; 52: 6940–6944.
- Sinicrope FA *et al.* Bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. *Cancer Res* 1995; 55: 237–241.
- King ED, Matteson J, Jacobs SC, Kyprianou N. Incidence of apoptosis, cell proliferation and bcl-2 expression in transitional cell carcinoma of the bladder: association with tumor progression. *J Urol* 1996; 155: 316–320.
- Saegusa M, Okayasu I. Bcl-2 is closely correlated with favorable prognostic factors and inversely associated with p53 protein accumulation in endometrial carcinomas: immunohistochemical and polymerase chain reaction/loss of heterozygosity findings. *J Cancer Res Clin Oncol* 1997; 123: 429–434.
- Piotti S *et al.* Bcl-2 protein expression in carcinomas originating from the follicular epithelium of the thyroid gland. *J Pathol* 1994; 172: 337–342.
- Leek RD *et al.* Bcl-2 in normal human breast and carcinoma, association with oestrogen receptor-positive, epidermal growth factor receptor-negative tumours and in situ cancer. *Br J Cancer* 1994; 69: 135–139.
- Moul JW *et al.* Protein expression of p53, bcl-2, and Ki-67 (MIB-1) as prognostic biomarkers in patients with surgically treated, clinically localized prostate cancer. *Surgery* 1996; 120: 159–167.
- Borre M, Nerstrøm B, Overgaard J. The dilemma of prostate cancer. A growing human and economic burden irrespective of treatment strategies. *Acta Oncol* 1997; 36: 681–687.
- Jacobs TW, Prioleau JE, Stillman IE, Schnitt SJ. Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. *J Natl Cancer Inst* 1996; 88: 1054–1059.
- Borre M *et al.* Association between in vivo iododeoxyuridine labeling, MIB-1 expression, malignancy grade and clinical stage in human prostate cancer. *APMIS* 1998; 106: 389–395.
- Byar DP, Mostofi FK. Carcinoma of the prostate: Prognostic evaluation of certain pathologic features in 208 radical prostatectomies. *Cancer* 1972; 30: 5–13.
- O'Malley FP *et al.* DNA heterogeneity in prostatic adenocarcinoma. *Cancer* 1993; 71: 2797–2802.
- Apakama I *et al.* bcl-2 overexpression combined with p53 protein accumulation correlates with hormone-refractory prostate cancer. *Br J Cancer* 1996; 74: 1258–1262.
- Stattin P *et al.* Bcl-2 immunoreactivity in prostate tumorigenesis in relation to prostatic intraepithelial neoplasia, grade, hormonal status, metastatic growth and survival. *Urol Res* 1996; 24: 257–264.
- Bauer JJ *et al.* Elevated levels of apoptosis regulator protein p53 and bcl-2 are independent prognostic biomarkers in surgically treated clinically localized prostate cancer. *J Urol* 1996; 156: 1511–1516.
- Bubendorf L *et al.* Prognostic significance of bcl-2 in clinically localized prostate cancer. *Am J Pathol* 1996; 5: 1557–1565.
- Moul JW *et al.* Protein expression of p53, bcl-2 and ki-67 (MIB-1) as prognostic biomarkers in patients with surgically treated, clinical localized prostate cancer. *Surgery* 1996; 120: 159–166.
- Lipponen P, Vesalainen S. Expression of the apoptosis suppressing protein bcl-2 in prostatic adenocarcinoma is related to tumor malignancy. *Prostate* 1997; 32: 9–15.
- Tu H, Jacobs SC, Borkowski A, Kyprianou N. Incidence of apoptosis and cell proliferation in prostate cancer: relationship with TGF- β 1 and bcl-2 expression. *Int J Cancer* 1996; 69: 357–363.
- Furuya Y *et al.* Expression of bcl-2 and the progression of human and rodent prostatic cancers. *Clin Cancer Res* 1996; 2: 389–398.



- 38 Byrne RL *et al*. The expression of waf-1, p53 and bcl-2 in prostatic adenocarcinoma. *Br J Urol* 1997; 79: 190–195.
- 39 Matsushima H *et al*. Bcl-2 expression on prostate cancer and its relationship to cell cycle and prognosis. *Int J Urol* 1996; 3.
- 40 Krajewska M *et al*. Immunohistochemical analysis of bcl-2, bax, bcl-x, and mcl-1 expression in prostate cancers. *Am J Pathol* 1996; 148: 1567–1576.
- 41 Berges RR *et al*. Implication of cell kinetic changes during the progression of human prostatic cancer. *Clin Cancer Res* 1995; 1: 473–480.

c-erbB-2 oncoprotein assay in ovarian carcinoma and its clinical correlation with prognostic factors

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Abstract

Overexpression of the c-erbB-2 oncoprotein has been detected in human adenocarcinoma of the breast, cervix and salivary gland, in all of which an association between the overexpression of the c-erbB-2 and a poor prognosis of the disease has been reported. However, the prognostic role of c-erbB-2 oncoprotein in ovarian carcinoma remains controversial. We measured c-erbB-2 oncoprotein with an enzyme-linked immunosorbent assay (ELISA). Patients with invasive ovarian cancer were found to have significantly higher median c-erbB-2 oncoprotein expression than patients with either benign ovarian cyst ($P = 0.002$) or control groups ($P = 0.001$). Overexpression of c-erbB-2 oncoprotein was found in seven (21.9%) of 32 epithelial ovarian cancers. Our results suggest that quantitative analysis of c-erbB-2 oncoprotein may be used to define the prognostic significance of ovarian carcinoma. © 1998 Elsevier Science Ireland Ltd. All rights reserved

Keywords: c-erbB-2 oncoprotein; Ovarian cancer; Enzyme immunoassay

1. Introduction

It has been established over recent decades that the molecular mechanisms implicated in the development and uncontrolled proliferation of cancers involve abnormalities of oncogenes and growth factor receptor systems. As our knowledge of the role of regulatory genes in oncogenesis increases, the quantitation of their expressed products will undoubtedly find increased clinical utility. Amplification of certain

oncogenes or an increase in their oncoproteins have been shown to have prognostic significance in several cancers [1–4].

c-erbB-2 oncoprotein is a 185 kDa membrane-bound glycoprotein. It is a receptor on the cytoplasmic membrane that is homologous to the epidermal growth factor receptor (c-erbB-1). The c-erbB-2 oncogene was independently discovered by several groups and consequently is referred to by various names, including HER-2 [5] and *neu* [6–8].

The c-erbB-2 molecule includes three domains, i.e. an extracellular domain, a short transmembrane domain and an intracellular domain [9–11]. The intracellular domain contains protein-tyrosine kinase activity capable of autophosphorylation. The portion of the c-erbB-2 oncogene which codes for this domain

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is 88% homologous to the corresponding part of the epidermal growth factor receptor gene and is also closely related to other known tyrosine kinase genes. It has been reported that overexpression of the c-erbB-2 gene in ovarian carcinoma is correlated with poor prognosis [12–15], although this association has been controversial [16–18].

c-erbB-2 oncoprotein has been investigated by several methods, such as radioligand binding assays, immunohistochemistry and multiparameter flow cytometry. However, until now there have been few enzyme immunoassay studies of c-erbB-2 oncoprotein on ovarian carcinoma. We recently reported the epidermal growth factor receptor in carcinoma of the uterine cervix by employing an enzyme immunoassay [19].

In this study, we evaluated the c-erbB-2 oncogene product with the enzyme immunoassay in fresh tissues of controls, benign ovarian cysts and epithelial ovarian carcinomas. We also examined the relationship between the results of c-erbB-2 oncoprotein assay and various prognostic parameters, including surgical stage, tumor size, lymph node involvement and histologic cell types.

2. Materials and methods

The fresh ovarian tissue samples used in this study were obtained from 52 patients treated at Yonsei Medical Center from January 1993 to November 1995. This included 32 invasive ovarian cancers, 10 benign ovarian cysts and 10 control cases who underwent hysterectomy and oophorectomy due to benign gynecological disease. Lymphadenectomy was performed in patients with epithelial ovarian carcinomas. Of 32 patients with ovarian cancer, 28 underwent total abdominal hysterectomy, bilateral salpingo-oophorectomy, supracolic omentectomy and pelvic-paraaortic lymphadenectomy, while the remaining four patients underwent the same procedures except for the paraaortic lymphadenectomy.

In this study, no patients had been treated with radiotherapy or chemotherapy prior to tissue sampling. Shortly after removal of ovarian tissue in the operating room, tissue specimens were stored at -70°C until they were processed and a portion of each tissue to be assayed for c-erbB-2 status was

examined to confirm the histological diagnosis of ovarian lesion.

For the tissue preparation, after stripping of blood and necrotic tissue, the obtained tissue samples were thawed on ice, placed in 10 volume of ice-cold receptor buffer (10 mM Tris-HCl (pH 7.4), 1.5 mM EDTA, 10% glycerol and 0.1% sodium azide) and homogenized. The resulting mixture was centrifuged at $1000\text{--}2000 \times g$ for 10 min at room temperature and the supernatant was recovered. The total protein in the prepared supernatant was measured by the method of Lowry et al. [20]. The c-erbB-2 oncoprotein enzyme immunoassay (c-erbB-2 EIA kit, Oncogene Science, New York, NY) which was used is a sandwich-type immunoassay involving a mouse monoclonal capture antibody [21] with a rabbit antiserum as a detector. The microtiter assay plate coated with anti-c-erbB-2 monoclonal antibody was incubated with specimen (tissue supernatant) and the standard. During this incubation, the receptor protein present in the specimen or the standard was bound to the solid phase and the unbound materials present in the specimen were removed by aspiration of fluid and washing of the plate. Anti-c-erbB-2 conjugated with horseradish peroxidase was incubated with the plate and if the receptor proteins were present in the specimen, the c-erbB-2 conjugates were bound to the receptors in the plate. Unbound conjugate was removed by aspiration and the plate was washed. The plate was then incubated with enzyme substrate solution (hydrogen peroxide and *O*-phenylenediamine) to develop color which reflected the amount of bound c-erbB-2 conjugate. The enzyme reaction was stopped by the addition of 2.5 N sulfuric acid and the intensity of the color developed was read using a spectrophotometer set at 490 nm. The intensity of the color formed was proportional to the concentration of receptors in the sample. A standard curve was obtained by plotting the c-erbB-2 concentration of the standards according to the absorbance and the values of the specimens were determined from the curve. The concentration of the c-erbB-2 was calculated in fmol/mg cytosol protein by dividing the concentration of receptors by the concentration of cytosol protein. Statistical analysis was carried out by non-parametric tests, including the Mann-Whitney *U*-test and the Kruskal-Wallis test. Differences were considered significant when the probability of error was below 5% ($P < 0.05$).

3. Results

The age of the study subjects ranged from 29 to 72 years with a mean of 54.6 years. The benign ovarian cyst group included six patients with serous cystadenoma, two patients with hemorrhagic corpus luteum cyst and two patients with endometriotic cyst. For the surgical stage distribution of ovarian carcinoma, patients with stage III ovarian carcinoma were the most numerous, accounting for 19 cases. According to the cell type, 17 cases were serous cystadenocarcinoma, eight were mucinous type, two were endometrioid type and two were clear cell carcinoma. When categorized by menstrual status, 21 cases of ovarian carcinoma were premenopausal and 11 cases were postmenopausal.

A summary of c-erbB-2 expression in control, benign cyst and ovarian carcinoma groups is shown in Table 1. Patients with ovarian carcinoma were found to have significantly higher median c-erbB-2 expression than the patients with benign cyst ($P < 0.05$) or control groups ($P < 0.05$). However, there was no significant difference in the c-erbB-2 status between the benign cyst and control groups. Overexpression (defined as a c-erbB-2 level exceeding 100 fmol/mg protein) of c-erbB-2 was found in seven (21.9%) of 32 ovarian cancers and in none of 10 benign cyst patients.

3.1. Relationship with initial and residual tumor size

The c-erbB-2 status in ovarian carcinomas of different initial tumor sizes, those greater than 20 cm and smaller than 20 cm, were compared to evaluate the tumor proliferation potential. Lesions of 20 cm and over had significantly higher receptor levels than

Table 1
c-erbB-2 oncoprotein status in control, benign cyst and carcinoma

	No. of cases	Positive rate (%)	c-erbB-2 (fmol/mg protein)		
			Mean	Median	Range
Control	10	0	32	33*	25–93
Benign cyst	10	0	42	32**	24–92
Carcinoma	32	21.9	148	66	19–912

* $P = 0.001$ (carcinoma versus control); ** $P = 0.002$ (carcinoma versus benign cyst).

Table 2

c-erbB-2 oncoprotein status according to initial and residual tumor size

	No. of cases	c-erbB-2 (fmol/mg protein)		
		Mean	Median	Range
Initial tumor size (cm)				
≥20	8	436	449*	119-912
<20	24	178	134	24-290
Residual tumor size (cm)				
≥2	7	131	45	28-622
<2	25	156	67	19-912

* $P = 0.027$.

those lesions under 20 cm in size ($P = 0.027$) (Table 2).

The relationship between the median c-erbB-2 concentrations in ovarian carcinoma and the residual tumor size is also shown in Table 2. There was no statistical difference between the residual tumor size and the c-erbB-2 expression in patients with epithelial ovarian cancer. Residual tumor size did not appear to affect c-erbB-2 oncoprotein expression.

3.2. Relationship with surgical stages

To evaluate the relationship between c-erbB-2 expression and surgical stages, a comparison of eight patients with stage I, two patients with stage II, 19 patients with stage III and three patients with stage IV was performed. With its correlation according to surgical stage, a trend of increased median c-erbB-2 oncoprotein concentrations was noted with each advancing stage, presenting as 62 fmol/mg protein in stage I, 186 fmol/mg protein in stage II, 223 fmol/mg protein in stage III and 417 fmol/mg protein in stage IV. However, due to the limited number of cases in various stages, there was no significant difference (Fig. 1).

3.3. Relationship with histological cell type

The receptor status of different histologic types of ovarian carcinoma was analyzed. The comparison of median c-erbB-2 expression according to serous, mucinous, endometrioid and clear cell type revealed 235, 178, 345 and 213 fmol/mg protein, respectively. However, there was no statistical significance (Fig. 2).

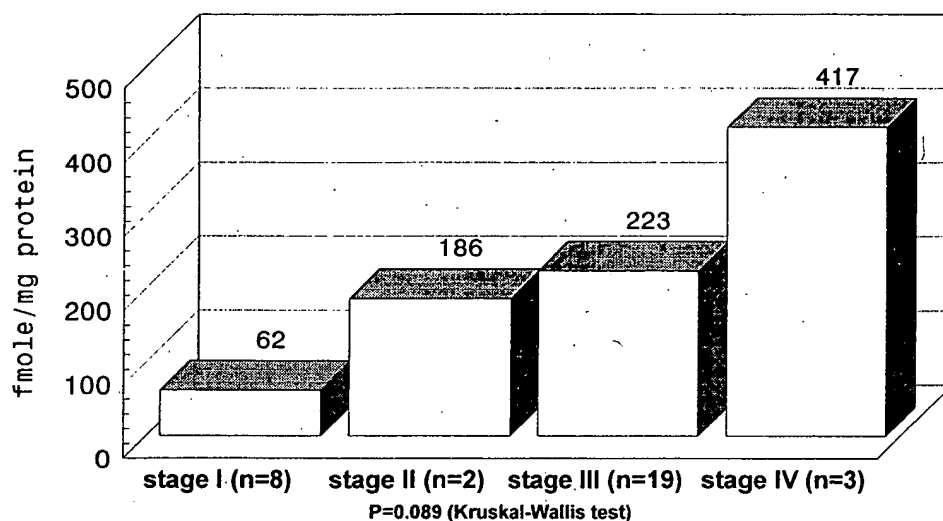


Fig. 1. Median c-erbB-2 oncoprotein expression in patients with ovarian carcinoma according to surgical stage.

3.4. Relationship with pelvic lymph node involvement

The relationship between median c-erbB-2 oncoprotein levels in epithelial ovarian carcinoma and pelvic lymph node involvement is demonstrated in Fig. 3. Ovarian cancer patients with pelvic lymph node metastasis were found to have a significantly higher median c-erbB-2 oncoprotein value than patients without pelvic lymph node involvement ($P < 0.05$).

4. Discussion

In the mid 1980s, the c-erbB-2 oncogene was revealed by several investigators. The *neu* oncogene was detected as a mutated transforming gene in neuroblastomas induced by ethylnitrosourea treatment of fetal rats [6-8]. The c-erbB-2 was a human gene discovered by its homology to the retroviral gene v-erbB [4,22,23]. *HER-2* was isolated by screening a

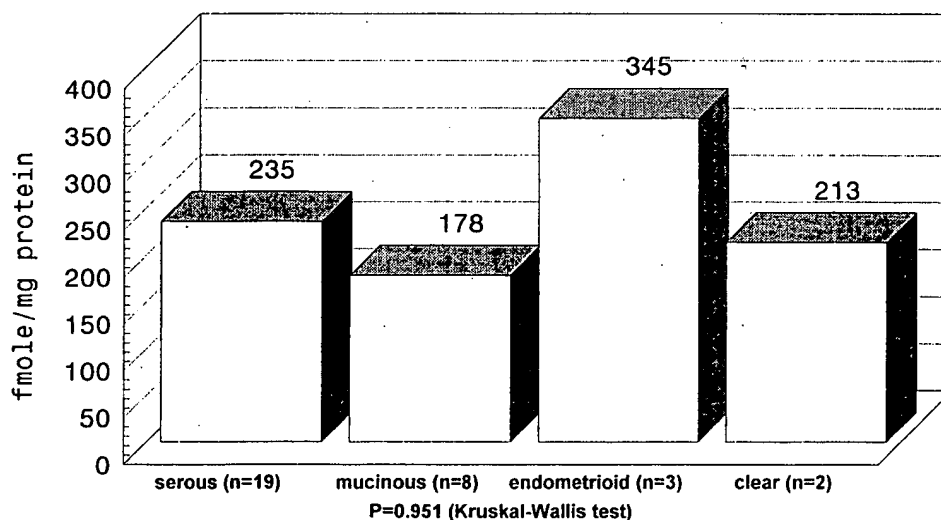


Fig. 2. Median c-erbB-2 oncoprotein expression in patients with ovarian carcinoma according to histological cell type.

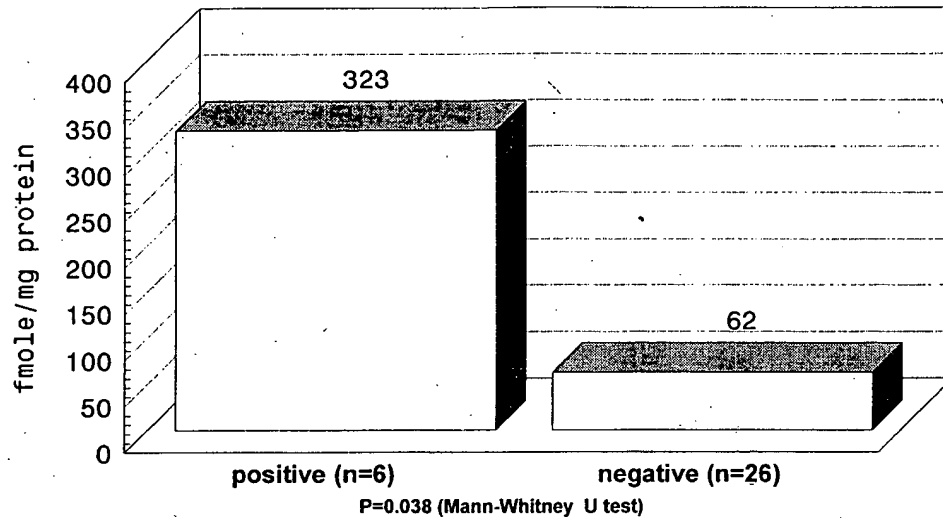


Fig. 3. Median c-erbB-2 oncoprotein expression in patients with ovarian carcinoma according to lymph node involvement.

human genomic DNA library for homology with *v-erbB* [5]. When the DNA sequences were subsequently determined, *c-erbB-2*, *HER-2* and *neu* were found to represent the same gene. The *c-erbB-2* oncogene is located on human chromosome 17q21 and encodes for *c-erbB-2* mRNA (4.6 kb), which translates *c-erbB-2* oncoprotein (p185). This oncoprotein is a glycoprotein which is a normal component of cytoplasmic membranes. The *c-erbB-2* molecule contains three domains, i.e. an extracellular domain, a short transmembrane domain and an intracellular domain [9–11]. The intracellular domain contains protein-tyrosine kinase activity capable of autophosphorylation. The portion of the *c-erbB-2* oncogene which codes for this domain is 88% homologous to the corresponding part of the epidermal growth factor receptor gene and is also closely related to other known tyrosine kinase genes.

There has been much experimental evidence that the *c-erbB-2* oncoprotein may be involved in the tumorigenesis of some neoplasia. Overproduction of *c-erbB-2* oncogene products can transform a cell line into a malignant phenotype [24]. Also, monoclonal antibodies against the *neu* protein inhibit the growth (in nude mice) of *neu*-transformed cell lines [25–27]. Unlike human breast cancers, where a variety of studies identifying *c-erbB-2* oncogene products have been applied, there have been few reports published on *c-erbB-2* oncoprotein found in ovarian carcinomas

correlated with clinical prognostic parameters and even fewer on benign ovarian tumors.

Several methods for investigating *c-erbB-2* oncoprotein in ovarian cancer have been applied, including the Western blot assay [12,13], the immunoprecipitation method [12,13], immunohistochemistry [12–18, 28–31] and flow cytometric analysis [32]. However, very few studies have examined the *c-erbB-2* oncoprotein in fresh tissues of epithelial ovarian cancer with an enzyme immunoassay. By contrast with other methods for detecting *c-erbB-2* oncogene production, quantitative measurement of *c-erbB-2* oncogene products can be obtained with the enzyme immunoassay. In addition, it requires only a small volume of tumor tissue and it can be performed in a short period [19].

Immunohistochemical techniques permitted good qualitative localization of oncoprotein products but these procedures often generate conflicting results according to polyclonal versus monoclonal antibodies, methods of tissue fixation and interpretation criteria [16–18].

The enzyme immunoassay technique was used in this study to detect *c-erbB-2* overexpression in 21.9% of ovarian carcinomas. *c-erbB-2* overexpression ranging as widely as 9–72% has been noted in ovarian carcinomas [12–18,28–30] [31–33]. Different techniques and diverse cut-off levels have been used resulting in a wide variety of reported *c-erbB-2* over-

expression. Another possible explanation for the wide variability of c-erbB-2 overexpression could be the inadequate selection of cases. As previously reported [18], significant differences may have existed if borderline ovarian tumors were included in the studies. Our study excluded tumors of low malignant potential. Because the c-erbB-2 levels of 10 control cases were less than 93 fmol/mg cytosol protein, we used a cut-off point of 100 fmol/mg cytosol protein to indicate the overexpression.

When we attempted to correlate our enzyme immunoassay data with clinical prognostic parameters in patients with ovarian carcinoma, there was no significant correlation of c-erbB-2 oncoprotein status between age, menopausal status, residual tumor size and histological cell types. This finding was consistent with previous reports using immunohistochemistry [14,15,34].

With regard to prognosis, some authors have reported that overexpression of c-erbB-2 oncoprotein correlates with shorter survival [12–15], while others reached the opposite conclusion based on different data [16–18]. Our study revealed many positive cases among the advanced stage tumors, large tumors and tumors with nodal metastasis. Therefore, it seems likely that the determination of quantitative changes of oncogene products will prove to be of clinical use in classifying tumors into different prognostic categories given that such changes at the molecular level may lead directly to alterations in tumor behavior. However, further study based on larger numbers of cases correlating c-erbB-2 oncoprotein status and complete follow-up survival data will help to confirm these findings.

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References

- [1] N.E. Hynes, D.F. Stern, The biology of erbB-2/neu/HER-2 and its role in cancer, *Biochim. Biophys. Acta* 1198 (1994) 165–184.
- [2] G.P. Cowley, J. Smith, B.A. Gusterson, Increased epidermal growth factor receptors on human squamous carcinoma cell lines, *Br. J. Cancer* 53 (1986) 223–229.
- [3] D.A. Fisher, J. Lakshmanan, Metabolism and effects of epidermal growth factor and related growth factors in mammals, *Endocr. Rev.* 11 (1990) 418–422.
- [4] R.C. King, M.H. Draus, S.A. Aaronson, Amplification of a novel v-erbB-related gene in a human mammary carcinoma, *Science* 229 (1985) 974–976.
- [5] L. Coussens, T.L. Yang-Feng, Y.U. Liao, L. Chen, A. Gray, J. McGrath, Tyrosine-kinase receptor with extensive homology to EGF receptor shares chromosomal location with new oncogene, *Science* 230 (1985) 1132–1139.
- [6] C.I. Bargmann, M.C. Hung, R.A. Weinberg, The new oncogene encodes an epidermal growth factor receptor-related protein, *Nature* 319 (1986) 226–230.
- [7] A.L. Schechter, D.F. Stern, L. Vaidyanathan, The neu oncogene: an erb-B-related gene encoding a 185 000-M tumor antigen, *Nature* 312 (1984) 513–519.
- [8] C. Shih, L.C. Padhy, M. Murray, R.A. Weinberg, Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts, *Nature* 290 (1981) 261–267.
- [9] L.C. Padhy, C. Shih, D. Cowing, R. Finkelstein, R.A. Weinberg, Identification of a phosphoprotein specifically induced by the transforming DNA of rat neuroblastomas, *Cell* 28 (1981) 865–871.
- [10] T. Yamamoto, S. Ikawa, T. Akiyama, K. Semba, N. Nomura, N. Miyajima, T. Sait, K. Toyoshima, Similarity of protein encoded by the human c-erbB-2 gene to epidermal growth factor receptor, *Nature* 319 (1986) 230–234.
- [11] T. Akiyama, C. Sudo, H. Ogawa, K. Totoshima, T. Yamamoto, The product of the human c-erbB-2 gene: a 185 kilodalton glycoprotein with tyrosine kinase activity, *Science* 232 (1986) 1644–1646.
- [12] D.J. Slamon, W. Godolphin, L.A. Jones, J.A. Holt, S.G. Wong, D.E. Keith, W.J. Lenin, S.G. Stuart, H. Udove, A. Ullrich, M.F. Press, Studies of the HER-2/neu protooncogene in human breast and ovarian cancer, *Science* 244 (1989) 707–712.
- [13] M.F. Press, L.A. Jones, W. Godolphin, C.L. Edwards, D.J. Slamon, Her-2/neu oncogene amplification and expression in breast and ovarian cancers, *Prog. Clin. Biol. Res.* 354A (1990) 209–221.
- [14] A. Berchuck, A. Kamel, R. Whitaker, B. Kerns, G. Olt, R. Kinney, J.T. Soper, R. Dodge, D.L. Clarke-Pearson, P. Marks, S. McKenzie, S. Yin, R.C. Bast Jr., Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer, *Cancer Res.* 50 (1990) 4087–4091.
- [15] E. Felip, J.M.D. Lampo, D. Rubio, M.T. Vidal, R. Colomer, Overexpression of c-erbB-2 in epithelial ovarian cancer: prognostic value and relationship with response to chemotherapy, *Cancer* 75 (1995) 2147–2152.
- [16] J.S. Haldane, V. Hird, C.M. Hughes, W.J. Gullick, c-erbB-2 oncogene expression in ovarian cancer, *J. Pathol.* 162 (1990) 231–237.
- [17] B. Kacinski, A.G. Mayer, B.L. King, D. Carter, S.K. Chambers, NEU protein overexpression in benign, borderline, and

- malignant ovarian neoplasms, *Gynecol. Oncol.* 44 (1992) 245–253.
- [18] T.P. Shingleton, T. Perrone, G. Oakley, G.A. Niehans, L. Carson, S.S. Cha, J.G. Strickler, Activation of c-erbB-2 and prognosis in ovarian carcinoma, *Cancer* 73 (1994) 1460–1466.
- [19] J.W. Kim, Y.T. Kim, D.K. Kim, C.H. Song, J.W. Lee, Expression of epidermal growth factor receptor in carcinoma of the cervix, *Gynecol. Oncol.* 60 (1996) 283–287.
- [20] O.H. Lowry, N.J.J. Rosebrough, A.L. Farr, Protein measurement with the folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–269.
- [21] A. Nugent, E. McDermott, K. Duffy, N. O'Higgins, J.J. Fennelly, M.F. Duffy, Enzyme-linked immunosorbent assay of c-erbB-2 oncoprotein in breast cancer, *Clin. Chem.* 38 (1992) 1471–1474.
- [22] S.I. Jukushige, K.I. Matsubara, M. Yoshida, Localization of a novel v-erbB-related gene, c-erbB-2, on human chromosome 17 and its amplification in a gastric cancer cell line, *Mol. Cell. Biol.* 6 (1986) 955–961.
- [23] K. Semba, N. Kamata, K. Toyoshima, T. Yamamoto, A c-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma, *Proc. Natl. Acad. Sci. USA* 82 (1985) 8497–8502.
- [24] P.P. DiFiore, J.H. Pierce, M.H. Kraus, O. Segatto, C.R. King, S.A. Aaronson, ErbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells, *Science* 237 (1987) 178–182.
- [25] H. Sasano, C.T. Garrett, D.S. Wilkinson, S. Silverberg, J. Comerford, J. Hyde, Protooncogene amplification and tumor ploidy in human ovarian neoplasms, *Hum. Pathol.* 21 (1990) 382–391.
- [26] A.M. Mileo, M. Fanuele, F. Battaglia, G. Scambia, C. Benedetti-Panici, E. Mattei, Preliminary evaluation of HER-2/neu oncogene and epidermal growth factor receptor expression in normal and neoplastic human ovaries, *Int. J. Biol. Markers* 7 (1992) 47–51.
- [27] M. Tal, M. Wetzler, Z. Josefberg, A. Deutch, M. Gutmar, D. Assaf, Sporadic amplification of the HER2/neu protooncogene in adenocarcinomas of various tissues, *Cancer Res.* 48 (1988) 1517–1520.
- [28] P.G. Natali, M.R. Nicotra, A. Bigotti, I. Benturo, K.J. Slamon, B.M. Fendly, A. Ullrich, Expression of the p185 encoded by HER2 oncogene in normal and transformed human tissues, *Int. J. Cancer* 45 (1990) 457–461.
- [29] K. Chang, I. Ding, F.G. Kern, M.C. Willingham, Immunohistochemical analysis of p53 and HER-2/neu proteins in human tumors, *J. Histochem. Cytochem.* 39 (1991) 1281–1287.
- [30] H.H. Gallion, D.E. Powell, L.W. Smith, J.K. Morrow, A.W. Martin, J.R. vanNagell, E.S. Donaldson, Chromosome abnormalities in human epithelial ovarian malignancies, *Gynecol. Oncol.* 38 (1990) 473–477.
- [31] J.D. Seidman, D.M. Frisman, H.J. Norris, Expression of the HER-2/neu proto-oncogene in serous ovarian neoplasms, *Cancer* 70 (1992) 2857–2860.
- [32] P.A. vanDam, D.G. Lowe, J.V. Watson, M. James, T. Chard, C.N. Hudson, J.H. Shepherd, Multiparameter flow-cytometric quantitation of epidermal growth factor receptor and c-erbB-2 oncoprotein in normal and neoplastic tissues of the female genital tract, *Gynecol. Oncol.* 42 (1991) 256–264.
- [33] M.C. Hung, X. Zhang, D.H. Yan, H.Z. Zhang, G.P. He, Aberrant expression of the c-erbB-2/neu protooncogene in ovarian cancer, *Cancer Lett.* 61 (1992) 95–103.
- [34] S.C. Rubin, C.L. Finstad, G.Y. Wong, L. Almadrones, M. Plante, K.O. Lloyd, Prognostic significance of HER-2/neu expression in advanced epithelial ovarian cancer; a multivariate analysis, *Am. J. Obstet. Gynecol.* 168 (1993) 162–169.

Immunohistochemical Analyses of Estrogen Receptor in Endometrial Adenocarcinoma Using a Monoclonal Antibody¹

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ABSTRACT

Immunohistochemical localization of estrogen receptor (ER) using specific monoclonal anti-human estrogen receptor antibody, H222, with an immunoperoxidase technique was performed on fresh frozen tissue derived from 100 endometrial adenocarcinomas. Immunohistochemical evaluation incorporated both intensity and distribution of staining. In all cases, H222 localized in the nucleus of target cells. A significant quantitative relationship was shown between histological score (H-Score) and the biochemical analysis of ER content in tissue homogenates ($r = 0.65$, $P = 0.00001$). Excellent sensitivity (92%) and specificity (93%) were observed for the comparison of H-Score to the biochemical assay. Significant ER localization was present in stromal and myometrial elements, component H-Score of which correlated weakly with component H-Scores of malignant epithelial elements. Divergent receptor localization in stromal and myometrial versus malignant epithelial elements suggests that biochemical assays of endometrial carcinoma specimens may not reflect cancer-relevant receptor content. The data presented here suggest that the immunoassay of ER using H222 monoclonal antibody provides additional histochemical information to complement conventional analyses of endometrial adenocarcinomas.

INTRODUCTION

While sex steroid receptor analysis plays a pivotal role in the evaluation of patients with breast cancer, its clinical application has not become as well established for gynecological neoplasms (1, 2). The standard quantitative biochemical methods for steroid receptor analysis, dextran-coated charcoal assay, and sucrose density gradient assay do not consistently predict hormonal responsiveness and prognosis in endometrial carcinomas even though high concentrations of specific receptor are often present. Uterine tissue components, such as stroma and myometrium, are frequently included in specimens submitted for estrogen receptor analysis, are known to contain substantial quantities of ER,³ and thus may obscure detection of the cancer-specific ER component most relevant to response (3). By contrast, normal nonepithelial components of the breast do not contribute significantly to receptor levels, thus simplifying interpretation of receptor content in breast cancer specimens.

The dextran-coated charcoal and sucrose gradient density analyses are accurate and reliable methods but are subject to certain limitations. Inherent in biochemical assays of tissue homogenates is an inability to distinguish the source or distribution of cells which have binding activity. Numerous attempts have been made at developing histochemical methods for receptor analysis using fluorescein steroid conjugates (4, 5) or anti-steroid antibodies (6, 7), but none heretofore has correlated

well with biochemical assays or clinical outcome (8-12).

The recent development by Greene *et al.* (13-15) and Miller *et al.* (16) of highly specific and sensitive monoclonal antibodies directed against human estrogen receptor provides a new approach to histochemical receptor localization. The availability of well-characterized reagents and a sensitive immunoperoxidase technique allow detection and amplification of the few receptor molecules present in individual cells (17, 18). We report the results of a study utilizing the monoclonal anti-human estrogen receptor antibody H222 to assess the relative contribution of cancer and noncancerous components of endometrial adenocarcinomas to total biochemical estrogen receptor content and compare the estrogen receptor content to tumor differentiation.

MATERIALS AND METHODS

Patient Population. Tissues were evaluated from 173 patients with endometrial adenocarcinoma seen at Duke University Medical Center from January 1, 1980, to December 31, 1983. All tissues were submitted for pathological examination and analyses of steroid receptor content. The histological criteria for inclusion are those defined by the International Federation of Gynecologists and Oncologists (19). One hundred cases had adequate tissue submitted for complete evaluation.

Of the 173 consecutive cases of endometrial adenocarcinoma, 73 cases were excluded from the study for the following reasons: in 32 cases there was inadequate tissue for complete analysis (included in this group were patients in whom diagnosis was by preoperative endometrial curettage); in 30 cases no residual tumor was seen on cryostat sections prepared from the remaining tissue available for immunohistochemical analysis (see below); 5 cases contained only necrotic tissue; and in 6 cases the tissue had undergone significant desiccation. The 100 remaining cases comprise a study group of 90 primary adenocarcinomas of the endometrium and 10 cases in which tissue was derived from metastatic foci.

The patients ranged in age from 30 to 92 years. Eighty patients were 56 years of age or older, 12 patients were between 50 and 55 years of age, and 8 patients were in the age range of 30 to 49 years.

Histological Classification and Grading. Histological grading was completed according to the International Federation of Gynecology and Obstetrics Cancer Committee. Grade 1 consisted of highly differentiated adenomatous carcinoma in which 100 to 75% of the tumor cells are differentiated with 0-25% of cells undifferentiated. Grade 2 is a differentiated adenocarcinoma with partly solid areas and Grade 3 is a predominantly solid growth pattern with poorly differentiated cells. Well-differentiated carcinoma was distinguished from atypical hyperplasia by the loss of polarity of the glandular epithelium, by irregular gland profiles with cribriform pattern and intraglandular bridging, and by relatively scant stroma in the areas of tumor.

Biochemical Estrogen Receptor Analyses. Estrogen receptor content of the tissues was analyzed using modifications of the methods described by Schrader *et al.* (20). Tissue was obtained fresh immediately, washed in buffer (10 mM Tris-HCl-5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-HCl-1.5 mM EDTA-1.0 mM thioglycerol-0.02% Na₂S₂O₅, pH 7.4) at 4°C, quick frozen in liquid nitrogen, and maintained at -80°C in airtight liquid nitrogen capsules until assayed. The frozen tissue was pulverized in liquid nitrogen using a Spex freezer mill (Spex Industries, Inc., Metuchen, NJ) at three-fourths power with

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³The abbreviations used are: ER, estrogen receptor; H-Score, histological score; PBS, phosphate-buffered saline.

a stainless steel impeller for 15 s (five 3-s cycles). The tissue powder was homogenized at a sample:buffer volume of 1:4 at 4°C using a Polytron (Brinkman Instruments, Westbury, NY) (setting 3) for 60 s (four 15-s cycles with 30-s cooling periods). Cytosol was prepared from the homogenate by centrifugation at $1,000 \times g$ for 10 min followed by $145,000 \times g$ for 1 h, both at 4°C. Endogenous unbound steroid was removed by 0.75% (w/v) Norit A (Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH) in 0.0025% dextran. The supernatant protein content was analyzed by the method of Bradford (21) and adjusted to 4–6 mg protein/ml. Sucrose density gradient and dextran-coated charcoal analyses were performed.

Sucrose Density Gradient Analysis. Aliquots of 250 μ l of cytosol were incubated for 4 h at 4°C with 1.6 pmol ^3H -hexalabeled estradiol (New England Nuclear, Boston, MA) containing a 100-fold excess of cold testosterone. Parallel control incubations also contained a 250-fold excess of cold diethylstilbestrol preincubated with cytosol for 5 min prior to incubation with the radioactive hormone. Two hundred μ l were layered on 10–28.5% isokinetic sucrose gradients and centrifuged to a $\omega^2 t$ of 157,417 in a Beckman SW 60 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 4°C (polyallomer tubes). Gradients were fractionated by upward displacement with 75% glycerol into 40 fractions. Radioactivity was determined in an LS-4000 Inter technique liquid scintillation counter (IN/US, Fairfield, NJ) using 10 ml Biofluor (New England Nuclear). Counting efficiency was determined by external standardization used for computer calculations. Sedimentation coefficients were determined by the use of ^{14}C -labeled bovine serum albumin (4.6S) or γ -globulin (7S) standards.

Dextran-coated Charcoal Analysis. The cytosol was diluted to 1–2 mg protein/ml, and 200- μ l aliquots were incubated for 16 h at 4°C in eight Kahn tubes containing 1.6–0.125 pmol ^3H estradiol and 100-fold concentration of cold testosterone. Parallel control incubations at each concentration, when adequate samples were available (or a single control incubation at 0.8 pmol ^3H estradiol when the sample was limited), contained a 250-fold excess of cold diethylstilbestrol, added immediately prior to the radioactive steroid. Non-protein-bound free steroid was removed by 0.75% (w/v) Norit A (dextran-treated) and bound radioactivity (not removed by the charcoal) was determined. Calculations of K_d (dissociation constant) and bound receptor were made by the methods of Woosley and Muldoon (22) and Scatchard (23) by means of an on-line LEM computer in an LS-4000 Inter technique liquid scintillation counter.

Immunohistochemical Estrogen Receptor Analysis. Portions of the tissue specimens which had been fresh frozen in liquid nitrogen for biochemical assays were prepared as serial 4- to 6- μ m cryostat sections mounted on poly-L-lysine-coated slides. The initial section was stained with hematoxylin-eosin for tissue diagnosis. Histological grade of the tumor was evaluated according to Federation of International Gynecologists and Oncologists criteria (19). Assignment of tumor type and grade was confirmed by review of formalin-fixed, paraffin-embedded, hematoxylin-eosin-stained sections taken from the same specimens.

Monoclonal antibody H222, developed against MCF-7 human breast cancer estrogen receptors by Dr. L. S. Miller, Abbott Laboratories (16), was selected for this study. This monoclonal antibody has been shown to be specific for human estrogen receptor from human endometrium and human breast cancer by several criteria (16, 17, 24). H222 recognizes a stable and well-conserved determination that is close to the steroid-binding site. The peroxidase-antiperoxidase method of Sternberger (18) for immunohistochemical localization was performed as follows.

1. Cryostat sections were placed in 3.7% formaldehyde-phosphate-buffered saline for 10 min. They were then transferred to a PBS bath until formalin fixation had been completed for all sections in a given run.
2. Slides were placed in cold absolute methanol at -15°C to -20°C for 4 min and then cold acetone at -15°C to -20°C for 1 min.
3. Slides were washed in PBS twice for 5 min each at ambient temperature.
4. Sections were treated with 2% normal goat serum in PBS for 15 min in a humidified chamber to reduce the nonspecific binding of bridging antibody (see below).

5. Sections were incubated with primary antibody (H222, 5 $\mu\text{g}/\text{ml}$, 33 pmol/ml in PBS) for 30 min at ambient temperature. Negative control slides of adjacent cryostat sections from each of the same tissues were incubated with control antibody (normal nonimmunized rat immunoglobulin in PBS) in place of the primary antibody for 30 min. Control slides of estrogen receptor-rich MCF-7 human breast cancer cells were incubated with primary control antibody.

6. Slides were washed in PBS twice for 5 min each.
7. Sections were incubated with bridging antibody (goat anti-rat immunoglobulin in PBS) for 30 min.
8. Slides were washed in PBS twice for 5 min each.
9. Peroxidase-antiperoxidase complex horseradish peroxidase-rat anti-horseradish peroxidase in PBS was applied to the sections for 30 min.

10. Slides were washed in PBS twice for 5 min each.
11. Slides were flooded with 8 mg diaminobenzidine and 16 ml hydrogen peroxide in PBS for 6 min in the dark. The diaminobenzidine- H_2O_2 solution had been prepared using minimal light and was used within 30 min.

12. Sections were rinsed in gently running tap water for 5 min, dehydrated in serial alcohols to xylene, and coverslipped with Permount without counterstaining.

In the presence of hydrogen peroxide, the tissue-bound peroxidase converts the diaminobenzidine chromagen to an insoluble brown reaction product which can be visualized with a light microscope. By this procedure, the minute quantity of estrogen receptor-monoclonal antibody complex is amplified through the use of the bridging antibody, peroxidase-antiperoxidase complex, and enzyme reaction products.

Scoring of Assays. Biochemical assays were summarized as fmol of specific estrogen binding per mg of tissue protein.

The immunohistochemical localization was scored in a semiquantitative fashion incorporating both the intensity and distribution of specific staining. Evaluations were recorded for each observed tissue component, *i.e.*, myometrium, stroma, benign epithelium, and malignant epithelium, as percentage of cells in each of four intensity categories. The intensity of specific staining was characterized as not present (0), weak but detectable above control (1+), distinct (2+), and very strong (3+). For each observed tissue component, a summary value we refer to as H-Score was calculated. This consists of a sum of the percentages of positively stained cells multiplied by a weighted intensity of staining

$$\text{H-Score} = \sum P_i (i + 1),$$

where P_i is the percentage of stained cells in each intensity category, and i is the intensity for $i = 1, 2, 3$. A total H-Score for the tissue section was derived as the sum of the component H-Scores weighted by the fraction of each component observed in the tissue section.

$$\begin{aligned} \text{Total H-Score} = & \text{Cancer H-Score} \times \frac{\% \text{ Cancer}}{100} \\ & + \text{Stromal H-Score} \times \frac{\% \text{ Stroma}}{100} \\ & + \text{Myometrial H-Score} \times \frac{\% \text{ Myometrium}}{100} \\ & + \text{Benign epithelial H-Score} \times \frac{\% \text{ benign epithelium}}{100} \end{aligned}$$

Biochemical assays were compared to the total tissue histological score as well as to its components.

Sections were evaluated independently by two observers and rereviewed by the initial observer. Intraobserver ($r = 0.983$, $P = 0.00001$) and interobserver differences for total H-Score were resolved by consensus evaluation ($r = 0.994$, $P = 0.00001$) for primary observer *versus* consensus total H-Scores.

Data Management and Analysis. Biochemical assay values and immunohistochemical assay values were coded separately in a blinded fashion and maintained as independent files in the Time Oriented

Record for Oncology System at the Duke Cancer Center Database until completion of each phase of the study. Receptor data for final analysis was derived from the six-point dextran-coated charcoal analysis values except for cases in which tissue quantity was limiting in which case total receptor observed on sucrose density gradient analyses was used. The comparability of these analyses for threshold evaluation and category assignment has been shown previously (25). Record identification numbers were used to match samples for the analysis. Accuracies, sensitivities, specificities, and predictive values were determined as described (26). Quantitative comparison of biochemical and H-Score values was done by correlation analysis. The significance of two-way tables was determined by χ^2 test or Fisher's exact test as appropriate. Significance of differences in assay values between groups was determined by the Mann-Whitney *U* test. Distributions were plotted as the cumulative proportion of observations with a value less than or equal to the corresponding value on the abscissa.

RESULTS

Immunohistochemical localization of estrogen receptor identified with monoclonal anti-human estrogen receptor H222 antibody by indirect immunoperoxidase technique resulted in specific staining in the nuclei of target cells (Figs. 1 and 2). Specific cytoplasmic staining was not observed. Control slides treated with normal nonimmunized rat immunoglobulin in place of primary antibody demonstrated only minimal background staining.

Estrogen receptor localization occurred in stromal and myometrial elements as well as the epithelial component. When the epithelial component bound antibody, a heterogeneous pattern was generally observed. Few cases were uniform in their staining pattern. Among stromal and myometrial elements, however, staining was usually uniform (Fig. 2).

Cancer was present in every specimen retained in the study whereas stroma was present in 83% and myometrium in 26% of cases. The distribution of component percentages in specimens is shown in Fig. 3, which shows broad variation in component distributions despite macroscopic "trimming" of the specimen on receipt to "maximize" tumor epithelial components. Ten % of specimens contained benign epithelial elements; no specimen contained over 30% benign epithelial tissue. Nine of the 10 were positive with H222 ranging from 125 to 370 in component H-Score. The tenth value was 70. Because

of their low frequency, benign epithelial elements were not further analyzed.

The relationship between the logarithm of the quantitative biochemical assay of tissue homogenate extract (fmol of radio-labeled estradiol bound per mg of protein) and the H-Score of the total tissue section is shown in Fig. 4 ($r = 0.65$). Tables 1 and 2 compare total tissue H-Score to the biochemical assay. A biochemical receptor content of ≥ 10 fmol/mg protein was considered positive when its dissociation constant was less than or equal to 1.0×10^{-9} . An H-Score of ≥ 75 was the threshold value for a "positive" immunohistochemical assay. Sensitivity of the total tissue H-Score was 92% and specificity was 93% relative to the biochemical assay. The overall accuracy of the H-Score was 92%.

Correlations of individual tissue component H-Scores to biochemical ER appeared to reflect the percentages of tissue components present. Thus the malignant epithelial component, which was the predominant portion of most specimens, correlated with the biochemical value merely as well as did the total tissue H-Score. Overall accuracy of the malignant H-Score to biochemical ER dropped to 87%. Myometrial and stromal elements had a much weaker relationship to biochemical ER. Sensitivity and specificity were likewise degraded when considering individual tissue components (Table 2).

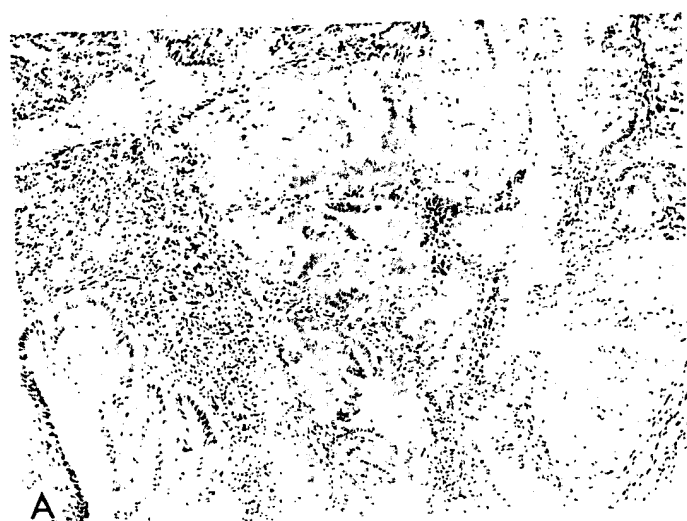
The relationship between the normal stromal and myometrial H-Score components and malignant H-Score is shown in Fig. 5. The myometrial component was moderately correlated with the cancer component ($r = 0.56$) but stromal component was only weakly correlated ($r = 0.30$).

Table 3 depicts the proportion of Grade I, Grade II, and Grade III tumors which were determined to be ER positive by each of the assays. Twelve % of Grade III tumors were ER positive by immunohistochemical evaluation of the malignant component only, whereas 35% were positive by biochemical ER assay. Twenty-three of 37 (62%) Grade II tumors were ER positive and 34 of 37 (92%) Grade I tumors were positive by malignant component H-Score compared to 76% of Grade II and 92% of Grade I tumors positive by biochemical ER.

The cumulative distribution function curves for biochemical ER in Fig. 6 shows an overlap in values between Grades I and II ($P = 0.50$), particularly for values above 50 fmol/mg protein, whereas Grade III values are shifted to significantly lower values



Fig. 1. Immunohistochemical localization of estrogen receptor using monoclonal anti-human estrogen receptor antibody H222 with the peroxidase-antiperoxidase technique. Sections were prepared from fresh frozen tissue. A, endometrial carcinoma with well-differentiated glands demonstrating intense nuclear localization of estrogen receptor. Staining is relatively weak in the stroma. B, control slide for A treated with normal nonimmunized rat immunoglobulin in place of the monoclonal anti-receptor antibody. Minimal background staining with absence of specific staining in target cells is observed. No counterstain, $\times 170$.



B

Fig. 2. Immunohistochemical localization of estrogen receptor-stromal-myometrial localization. In A, note the relatively homogeneous nuclear staining of the myometrium and stromal cells, which in contrast to the case in Fig. 1 are more intensely stained than the majority of the epithelial elements (see Fig. 5). Also note the heterogeneity of staining intensity in the glandular epithelium. In B, again, substituting control antibody for the primary antibody yields only minimal background staining. No counterstain, $\times 170$.

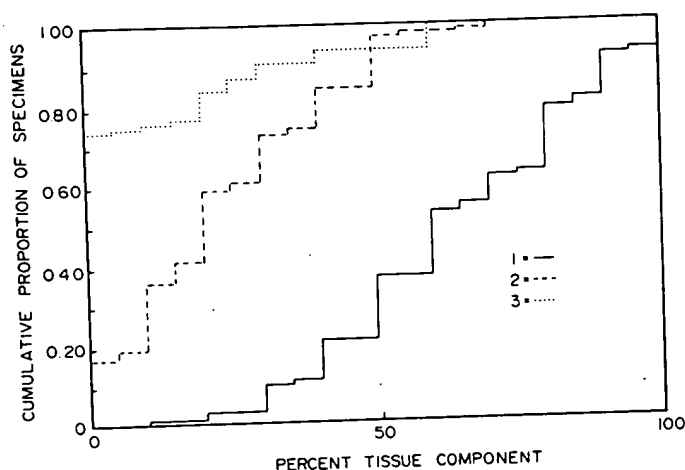


Fig. 3. Proportion of tissue components found in endometrial carcinoma specimens. Line 1, cancer component; Line 2, stromal component; Line 3, myometrial component. Cancer was present in all cases with only 37% of the specimens being composed of $\leq 50\%$ cancer. Some degree of benign epithelial elements were present in 10% of specimens.

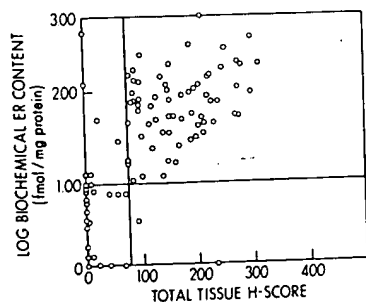


Fig. 4. Comparison of biochemical estrogen receptor content to total tissue histological score (H-Score). A linear relationship is observed between the logarithm of the quantitative biochemical assay of tissue homogenates (fmol of radiolabeled estradiol bound per mg protein) and the immunohistochemical H-Score of the total tissue section (intensity and proportion of cells showing positive localization of estrogen receptor). Six data points are at 0.0 ($r = 0.65$).

($P = 0.00002$, I versus III; $P = 0.0003$, II versus III). By contrast, the curves for the malignant epithelium (Fig. 7) show a highly significant separation between each of the curves ($P = 0.00002$, I versus II; $P = 0.00002$, II versus III).

In contrast to the relationship found between tumor grade

Table 1 Comparison of H-Score to total tissue homogenate extract biochemical ER content^a

H-Score	Biochemical ER		Total
	+	-	
Total tissue			
+	65	2	67
-	6	27	33
Total	71	29	
Malignant epithelium			
+	59	1	60
-	12	28	40
Total	71	29	
Myometrium			
+	19	2	21
-	3	2	5
Total	22	4	
Stroma			
+	29	2	31
-	36	16	52
Total	65	18	

^a Biochemical assay positive if ≥ 10 fmol/mg protein; immunohistochemical assay positive if H-Score ≥ 75 .

Table 2 Comparison of immunohistochemical assay to biochemical ER assay^a

Component	N	Accuracy (%)	Sensitivity (%)	Specificity (%)	Predictive value (%)	
					+	-
Total tissue (H-Score ≥ 75)	100	92.0	91.5	93.1	97.0	81.3
Malignant epithelium (H-Score ≥ 75)	100	87.0	83.1	96.6	98.3	70.0
Myometrium (H-Score ≥ 75)	26	80.8	86.4	50.0	90.5	40.0
Stroma (H-Score ≥ 75)	83	54.2	44.6	88.9	93.5	30.8

^a Biochemical assay positive if ≥ 10 fmol/mg protein; immunohistochemical assay positive if H-Score ≥ 75 .

and cancer component H-Score, the stromal and myometrial component H-Scores bore no relationship to the histological grade of the accompanying tumor (Fig. 8).

Histological patterns of the 100 cases of endometrial carcinomas included 77 adenocarcinomas with no special features, 15 papillary adenocarcinomas, 4 adenosquamous carcinomas, 2 adenoacanthomas, and 2 clear cell carcinomas. Positive localization of ER was not observed in the squamous elements of

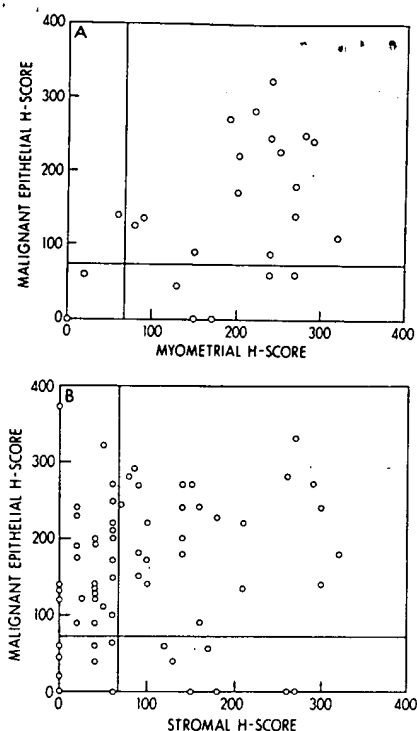


Fig. 5. Comparison of immunohistochemical scores (H-Score) for normal uterine component tissues to malignant epithelial component. A, cancer component versus myometrium. Three data points are at 0, 0 ($n = 26$, $r = 0.56$). B, cancer component versus stroma. Eight data points are at 0, 0 ($n = 83$, $r = 0.30$).

Table 3 Comparison of biochemical and immunohistological ER assays to tumor differentiation^a

Assay	Grade I (N = 37)	Grade II (N = 37)	Grade III (N = 26)
Biochemical ER (≥ 10 fmol/mg protein)	34 (92) ^b	28 (76)	9 (35)
Total tissue (H-Score ≥ 75)	35 (95)	26 (70)	6 (23)
Malignant epithelium (H-Score ≥ 75)	34 (92)	23 (62)	3 (12)

^a Biochemical assay positive if ≥ 10 fmol/mg protein; immunohistochemical assay positive if H-Score ≥ 75 .

^b Numbers in parentheses, percentage.

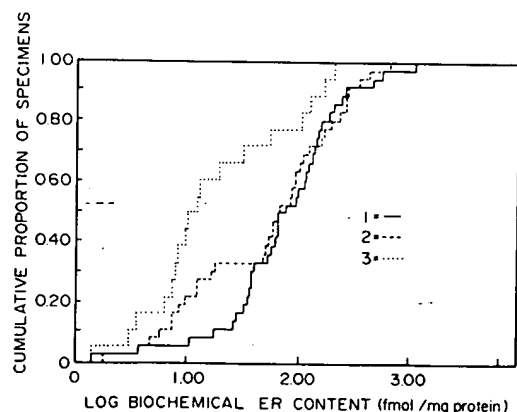


Fig. 6. Biochemically determined estrogen receptor content by degree of tumor differentiation. Line 1, Grade I; Line 2, Grade II; Line 3, Grade III. Ordinate, cumulative proportion of tumors for each Grade which have receptor contents less than or equal to the value on the abscissa. (1 versus 2, $P = 0.50$; 1 versus 3, $P = 0.00002$; 2 versus 3, $P = 0.0003$).

these tumors. There were no other characteristic differences in intensity, pattern, or distribution of immunohistochemically localized estrogen receptor observed among other histological variants.

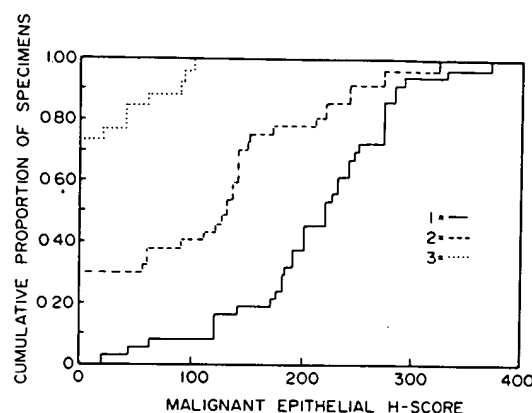


Fig. 7. Malignant epithelial H-Score by degree of tumor differentiation. Line 1, Grade I; Line 2, Grade II; Line 3, Grade III. Ordinate, cumulative proportion of tumors for each Grade which have H-Scores less than or equal to the value on the abscissa (1 versus 2, $P = 0.00002$; 1 versus 3, $P < 0.00001$; 2 versus 3, $P = 0.00002$).

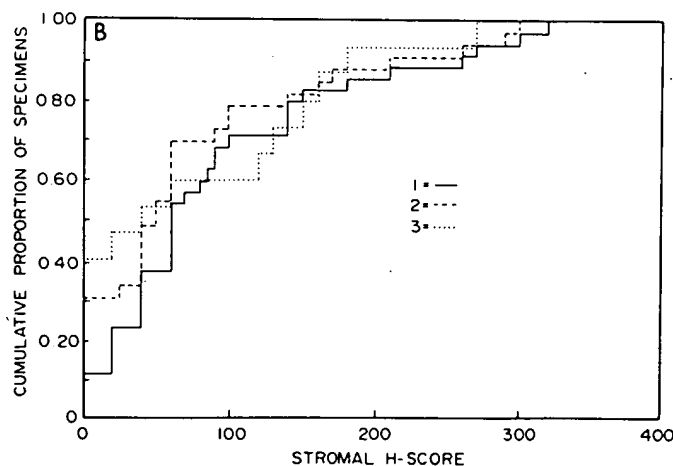
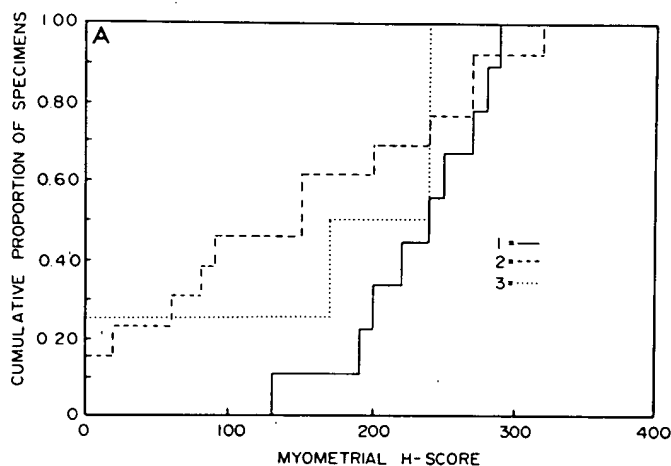


Fig. 8. H-Score of normal uterine tissue components by degree of tumor differentiation. Line 1, Grade I; Line 2, Grade II; Line 3, Grade III. A, myometrial component (1 versus 2, $0.05 < P < 0.1$; 1 versus 3, 2 versus 3, $P > 0.1$). B, stromal component (1 versus 2, 1 versus 3, 2 versus 3, $p > 0.1$).

DISCUSSION

Immunohistochemical analysis of estrogen receptor in endometrial adenocarcinoma, using H222 monoclonal anti-human estrogen receptor antibody with an immunoperoxidase technique, resulted in localization of ER principally in the nuclei of target cells. This nuclear localization of estrogen receptor is in agreement with other reports using monoclonal

antibody immunohistological techniques (24, 27, 28). Earlier studies had also suggested that receptor is predominantly located in the nucleus (29-31). Welshons *et al.* (32) used a novel approach in which, after estrogen receptor-rich rat pituitary GH₃ cells had been fractionated with cytochalasin B, the vast majority of unoccupied estrogen receptor was associated with nucleoplast rather than cytoplasm fraction. These findings suggest modification of the "two-step" mechanism of estrogen action (33, 34). What may be measured as cytosol receptor by standard biochemical assays may represent relative extraction from the nuclear compartment as a result of cell disruption during tissue homogenization (28, 29). It is likely that small amounts of receptor are present in the cytoplasm at sites of receptor synthesis which are not detected by immunohistochemical methods, but it seems reasonable from the data that the vast majority of receptor resides in the nucleus. Although modification of the standard model for estrogen receptor action seems to be indicated, its fundamental premise still holds; *i.e.*, activated steroid-receptor complexes exert their effects through interaction with the nuclei of target cells (35-37).

Comparison of biochemical assays of tissue homogenates for estrogen receptor values to the immunohistochemical assay on total tissue sections of endometrial carcinoma resulted in a correlation coefficient, accuracy, sensitivity, specificity, and predictive values with respect to biochemical ER superior to those observed by comparison to individual tissue components. While stromal and myometrial components were strongly related to total H-Score (as they must be since they entered into its calculation), they were only weakly correlated to the biochemical ER. This observation suggests that the normal tissue contribution to biochemical ER may be quantitatively less than that from the cancer component, while still significant, particularly in which the epithelium was ER poor.

A smaller proportion of Grade III tumors were shown to be ER positive by immunohistochemical analysis of the cancer component only than by biochemical assay of tissue homogenates, again reflecting the potential of stromal and myometrial elements to confound the biochemical assay of the tissue homogenates. Thus the cancer-specific H-Score might be anticipated to provide a discriminant of prognostic and therapeutic utility complementary to that of the biochemical assay.

A critical finding of this study is the poor correlation of receptor localization scores (H-Score) in stromal-myometrial elements to malignant epithelial element H-Score (Fig. 5). This observation emphasizes the suggestion that biochemical assays of total tissue homogenates may not accurately reflect estrogen receptor content of the cancer component, which is the principal element relevant to treatment response and prognosis. Inasmuch as nonmalignant components often may be significant in quantity, they may be expected to confound the prediction of biological response of the malignant component from biochemical receptor assays.

The correlation observed between biochemical assays and the immunohistological assay for estrogen receptor (Fig. 4) underscores the importance of incorporating both the percentage of positively stained cells and the intensity of individual cell staining in the evaluation of any histological receptor localization method. The intensity of staining appears to reflect the number of receptor sites, which may vary from 1,000 to 20,000 sites per cell (38). Fig. 4 and Tables 1-3 reflect the excellent sensitivity and specificity of this scoring technique at relatively low levels of estrogen receptor binding (10 fmol/mg protein).

An observation of particular note is the heterogeneity of staining in malignant epithelium compared to the relatively

homogeneous localization of ER in benign epithelium, stroma, and myometrium. Similar heterogeneity in breast cancers was noted by others (27). Whether this heterogeneity in staining reflects polyclonal origin of the tumors or asynchrony of receptor expression due to physiological factors requires further investigation. Press *et al.* (28) demonstrated that immunohistochemical localization of ER in human endometrium varied with cell type and with the menstrual cycle. They also noted the absence of specifically stained mitotic figures, suggesting that histological detection of ER may not be possible during some phases of the cell cycle.

Immunohistochemical localization of estrogen receptor using H222 monoclonal antibody appears to circumvent some of the complicating factors of benign epithelial, stromal, and myometrial contribution to biochemical receptor analyses of tissue homogenates. Such analyses may provide information that complements quantitative biochemical receptor analyses, pathological features, and tumor staging in the evaluation of endometrial adenocarcinomas including study of stromal-epithelial relationships in the biology of these tumors. The present study will serve as a data base for establishment of the possible significance of the differences observed in the behavior of these tumors with long term clinical follow-up. The observations reported should serve to alert others to the probable need to discriminate component contributions to total receptor in such studies.

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REFERENCES

- McCarty, K. S., Jr., Lubahn, D. B., and McCarty, K. S., Sr. Oestrogen and progesterone receptors: physiological and pathological considerations. *Clin. Endocrinol. Metab.*, 12: 133-154, 1983.
- Roberts, D. K., Van Sickle, M., and Kelly, R. W. The role of sex steroid receptors in obstetrics and gynecology. *Obstet. Gynecol. Annu.* 12: 61-78, 1983.
- Evans, L. H., Martin, J. D., Hahnel, R. Estrogen receptor concentration in normal and pathological human uterine tissues. *J. Clin. Endocrinol. Metab.* 38: 23-32, 1974.
- Lee, S. H. Cancer cell estrogen receptor of human mammary carcinoma. *Cancer (Phila.)*, 44: 1-12, 1979.
- Lee, S. H. Cellular estrogen and progesterone receptor in mammary carcinoma. *Am. J. Clin. Pathol.* 73: 323-329, 1980.
- Farley, A. L., O'Brien, T., Moyer, D., Taylor, C. R. The detection of estrogen receptors in gynecologic tumors using immunoperoxidase and the dextran-coated charcoal assay. *Cancer (Phila.)*, 49: 2153-2160, 1982.
- Pertschuk, L. P., Tobin, E. H., Brigati, B. J., Kim, D. S., Bloom, N. D., Gaetjens, E., Berman, P. J., Carter, A. C., Degenschein, G. A. Immunofluorescent detection of estrogen receptors in breast cancer; comparison with dextran-coated charcoal and sucrose gradient assays. *Cancer (Phila.)*, 41: 907-911, 1978.
- Chamness, G. C., Mercer, W. D., and McGuire, W. L. Are histochemical methods for estrogen receptor valid? *J. Histochem. Cytochem.*, 28: 792-798, 1980.
- Chamness, G. C., McGuire, W. L. Questions about histochemical methods for steroid receptors. *Arch. Pathol. Lab. Med.*, 106: 53-54, 1982.
- McCarty, K. S., Jr., Woodard, B. H., Nichols, D. E., Wilkinson, W., McCarty, K. S., Sr. Comparison of biochemical and histochemical techniques for estrogen receptor analyses in mammary carcinoma. *Cancer (Phila.)*, 46: 2842-2845, 1980.
- McCarty, K. S., Jr., Hiatt, K. B., Budwit, D. A., Cox, E. B., Leight, G., Reintgen, D., Georgiade, G., McCarty, K. S., Sr., Seigler, H. F. Clinical response to hormone therapy correlated with estrogen receptor analyses. Biochemical vs. histochemical methods. *Arch. Pathol. Lab. Med.* 108: 24-26, 1984.
- Panko, W. B., Mattioli, C. A., Wheeler, T. M. Lack of correlation of a histochemical method for estrogen receptor analysis with the biochemical assay results. *Cancer (Phila.)*, 49: 2148-2152, 1982.
- Greene, G. L., Fitch, F. W., Jensen, E. V. Monoclonal antibodies to estrogen

- philin: probes for the study of estrogen receptors. *Proc. Natl. Acad. Sci. USA* 77: 157-161, 1980.
14. Greene, G. L., Nolan, C., Engler, J. P., and Jensen, E. V. Monoclonal antibodies to human estrogen receptor. *Proc. Natl. Acad. Sci. USA*, 77: 5115-5119, 1980.
15. Greene, G. L., Jensen, E. V. Monoclonal antibodies as probes for estrogen receptor detection and characterization. *J. Steroid Biochem.* 16: 353-359, 1982.
16. Miller, L. S., Tribby, I. I. E., Miles, M. R., Tomita, J. T., Nolan, C. Hybridomas producing monoclonal antibodies to human estrogen receptor. *Fed. Proc.*, 41: 520, 1982.
17. Greene, G. L. Application of immunocytochemical techniques to the analysis of estrogen receptor structure and function. In: G. Litwack (ed.), *Biochemical Actions of Hormones*, Vol. 3, pp. 207-239. New York: Academic Press, 1984.
18. Sternberger, L. A. *Immunocytochemistry*, pp. 104-169. New York: John Wiley and Sons, Inc., 1979.
19. Novak, E. R., Woodruff, J. D. *Gynecologic and Obstetric Pathology with Clinical and Endocrine Relations*, pp. 198-199. Philadelphia: W. B. Saunders Company, 1974.
20. Schrader, W. T., Smith, R. G., Coty, W. A. Steroid hormone receptor purification and characterization. In: B. W. O'Malley and W. T. Schrader (eds.), *Laboratory Methods Manual for Hormone Action and Molecular Endocrinology*, pp. 2.1-2.58. Houston, TX: Baylor College, 1978.
21. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann. Biochem.*, 72: 248-254, 1976.
22. Woosley, J. T., and Muldoon, T. G. Use of the direct linear plot to estimate binding constants for protein-ligand interactions. *Biochem. Biophys. Res. Commun.* 71: 115-160, 1976.
23. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51: 660-672, 1949.
24. Press, M. F., Greene, G. L. An immunocytochemical method for demonstrating estrogen receptor in human uterus using monoclonal antibodies to human estrophilin. *Lab. Invest.* 50: 480-486, 1984.
25. Smith, R. G. Quality control in steroid hormone receptor assays. *Cancer (Phila.)*, 46: 2946-2949, 1980.
26. Galen, R. S., Gambino, G. S. R. *Beyond Normality: the Predictive Value and Efficiency of Medical Diagnosis*, pp. 1-14. New York: John Wiley and Sons, Inc., 1975.
27. King, W. J., Greene, W. L. Monoclonal antibodies localize estrogen receptor in the nuclei of target cells. *Nature (Lond.)*, 307: 745-747, 1984.
28. Press, M. F., Nousek-Geobl, N., King, W. J., Herbst, A. L., Greene, G. L. Immunohistochemical assessment of estrogen receptor distribution in the human endometrium throughout the menstrual cycle. *Lab. Invest.* 51: 495-503, 1984.
29. Linkie, D. M., and Siiteri, P. K. A re-examination of the interaction of estradiol with target cell receptors. *J. Steroid Biochem.*, 9: 1071-1078, 1978.
30. Sheridan, P. J., Buchanan, J. M., Anselmo, V. C., Martin, P. M. Equilibrium: the intracellular distribution of steroid receptors. *Nature (Lond.)*, 282: 579, 1979.
31. Stumpf, W. E. Subcellular distribution of ³H-estradiol in rat uterus by quantitative autoradiography: a comparison between ³H-estradiol and ³H-norethynodrel. *Endocrinology* 83: 777-782, 1968.
32. Welshons, W. V., Lieberman, M. E., Gorski, J. Nuclear localization of unoccupied oestrogen receptors. *Nature (Lond.)*, 307: 747-748, 1984.
33. Gorski, J., Toft, D., Shyamala, G., Smith, D., Notides, A. Hormone receptors: studies on the interaction of estrogen with the uterus. *Recent Prog. Horm. Res.* 24: 45-80, 1968.
34. Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W., DeSombre, E. R. A two-step mechanism for the interaction of estradiol with rat uterus. *Proc. Natl. Acad. Sci. USA* 59: 632-638, 1968.
35. Jensen, E. V., Greene, G. L., Closs, L. E., DeSombre, E. R., Nadj, M. Receptors reconsidered: a 20-year perspective. *Recent Prog. Horm. Res.* 38: 1-40, 1982.
36. Jensen, E. V. Intracellular localization of estrogen receptors: implications for interaction mechanism. *Lab. Invest.*, 51: 487-488, 1984.
37. Schrader, W. T. New model for steroid hormone receptors? *Nature (Lond.)*, 308: 17-18, 1984.
38. McCarty, K. S., Jr., and McCarty, K. S., Sr. Steroid hormone receptors in the regulation of differentiation. *Am. J. Pathol.* 86: 705-744, 1977.